THE UNIVERSITY OF ALBERTA

STREPTOMYCIN ADENYLYLTRANSFERASE-MEDIATED DIHYDROSTREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1978

ABSTRACT

The mechanism of resistance to dihydrostreptomycin of E. coli strains possessing a streptomycin adenylyltransferase (AAD-(3")), an R factor-determined enzyme, was inferred from evidence accumulated pertaining to a possible cellular localization of the enzyme, the effect of the enzyme on the energy-dependent accumulation of dihydrostreptomycin and the intracellular fate of modified drug. AAD-(3") was found to maintain an association with the bacterial cell membrane of sphaeroplasts. Its release during the preparation of pure membrane fractions indicates that the relationship is a relatively weak one. The enzyme lies on the external side of the membrane permeability barrier since impermeable chemical reagents can modify the enzyme in whole sphaeroplasts. From this position it mediates a reduction in the total accumulation of dihydrostreptomycin in cells by eliminating or reducing, depending on the drug concentration, the last of three phases of drug accumulation. This phase has been documented to correspond with the binding of drug by ribosomes of sensitive cells and also the events leading to cell death. By reducing the rate of transport of streptomycin in R factor-containing organisms resistance could be increased indicating that a balance exists between the rates of transport and inactivation which ultimately determines levels of susceptibility. Drug accumulated energetically in R+ and R- cells, at drug concentrations intermediate to the MICs of the strains, is cellularly distributed in an identical fashion. Under these conditions R+ cells, as opposed to R- cells, accumulate modified drug and show no signs of a third phase of uptake. Though autoradiographic analysis suggests that most of the drug is envelope-associated, drug can not be found associated with the membrane fractions of either cell type upon their purification. Thus, a loose association of the two is inferred.

The evidence acquired to date suggests that drug is intercepted early during its transport and modified by the streptomycin adenylyltransferase. R+ cells accumulate drug normally otherwise (in the manner of sensitive cells) throughout the first two phases of uptake: active drug superficially (1^{st} phase) and inactivated drug energetically (2^{nd} phase). The third phase of drug accumulation and the accompanying effects initiated by the binding of drug to the ribosomes are absent in R+ cells only because adenylylated dihydrostreptomycin is not capable of binding to ribosomes. An interruption of drug transport to these sensitive sites of R+ cells is not apparent.

ACKNOWLEDGMENTS

For their willing and earnest assistance throughout this investigation I would like to extend my sincerest appreciation to Dr. K. Roy of the Microbiology Department and Dr. M. S. Shahrabadi, Dr. R. Whitehouse and R. Sherburne of the Medical Bacteriology Department.

With no less gratitude may I thank the members of both departments who have indirectly supported me throughout my graduate program. Special thanks go to S. Fatemi Nainie and R. Bissonnette.

Foremost, I would like to express my gratitude to Dr. L. E. Bryan for his patient guidance and his readiness to discuss and to contribute to the arguments forwarded in this work while acting in his supervisory role. In Dr. Bryans absence during his sabbatical year, Dr. M. Pickard offered his most capable assistance and discussions shared with him have been more than appreciated.

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DHS	- dihydrostreptomycin
AMP-DHS	- adenylylated-dihydrostreptomycin
EDTA	- ethylene diamine tetraacetate
ATP	- adenosine triphosphate
EU	- enzyme unit
PC	- paper chromatography
MIC	- minimum inhibitory concentration
Tris	- Tris (hydroxymethyl) aminomethane
cpm	- counts per minute
sp. act.	- specific activity
A_x, OD_x	- absorbance, optical density at xnm wavelength
R factor	- resistance transfer factor
R+	- with R factor
R-	- without R factor
Str^r	- phenotypically streptomycin resistant by virtue of a single-step mutation
Str^s	- streptomycin sensitive (used in the same context as R-)
prot.	- protein

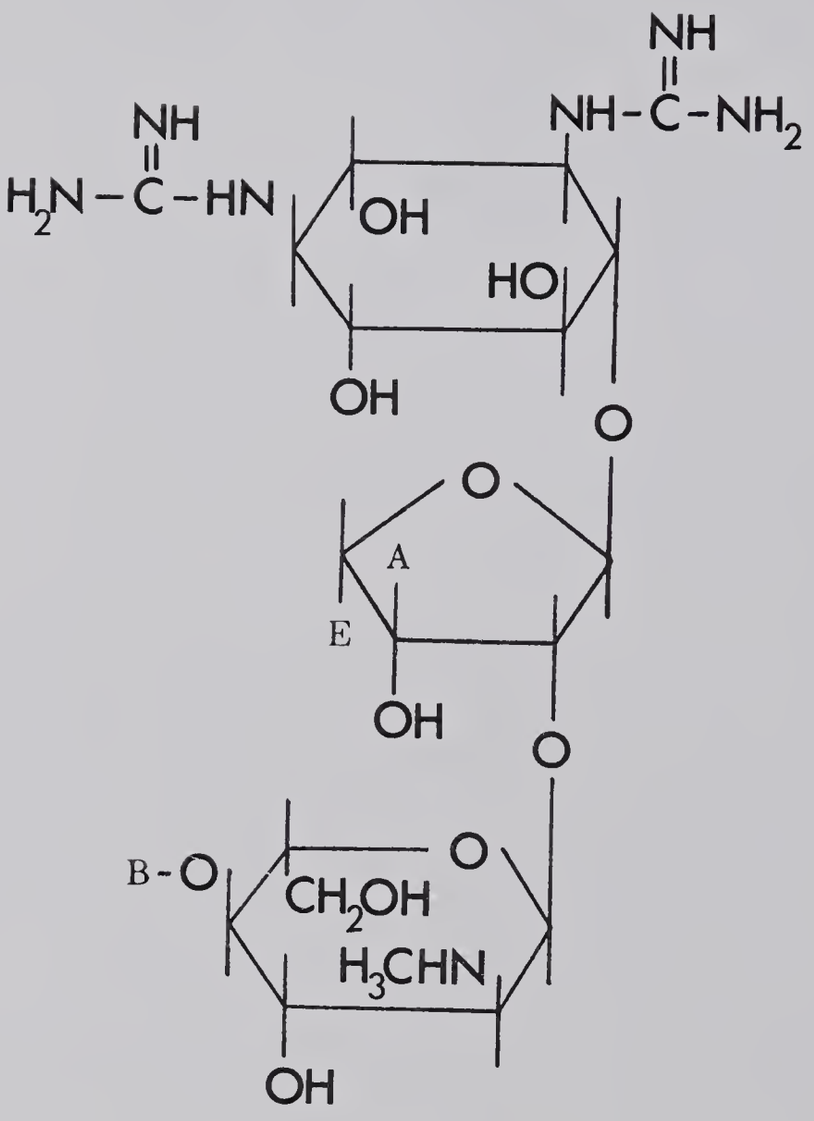
INTRODUCTION AND LITERATURE REVIEW

Antibiotics and the ensuing age of antibiotic chemotherapy evolved from the serendipitous discovery of penicillin by Alexander Fleming in 1929 and its later development as a chemotherapeutic agent in the 1940s. The practice of chemotherapy was not new. Paul Ehrlich had established its feasibility in the early 1900s although with limited personal success. The discovery of the sulphonamide drugs represents the great achievement arising from his primordial work. Then the natural antagonism some microorganisms displayed towards others, such as had been seen by the penicillin producers, stimulated the deliberate screening of organisms in an effort to isolate and develop further antibiotics of clinical efficacy and acceptability. The first success of pronounced clinical significance followed in 1944 when, under the auspices of S. A. Waksman who had invested some thirty years in the study of actinomycetes, streptomycin was discovered in the filtrates of one of these soil microorganisms. Like penicillin, streptomycin achieved widespread use before it was understood how the drug acted and why certain bacteria were affected and others were not (Epstein and Williams, 1946).

Today the characteristics of penicillin and penicillin action are comparatively well defined. In contrast, a debate spanning decades continues regarding the mechanism of action of streptomycin. Not clearly understood either are the mechanisms of resistance to the drug though the means by which these are acquired by bacteria are known. This knowledge is requisite to an orderly and efficient search for streptomycin derivatives of potentiated activity and also for the advancement of therapeutic practices capable of overcoming naturally resistant microbes without being deleterious to the host. A great deal has been achieved with penicillin

in this respect. Perhaps more importantly, a well-characterized streptomycin could be an invaluable tool to be engaged in investigations of other biological phenomena. A study of the relationship between streptomycin and the bacterial cell membrane, eventually leading to an improved appreciation of this vital substructure, is one possibility.

Streptomycin is an aminoglycoside. Chemically this family of antibiotics is characterized by the presence of aminosugars and aminocyclitol moieties. Differentiation into two subclasses is based on whether the molecules contain either streptidine or deoxystreptamine residues. With few exceptions they are the products of Streptomyces species and they share functional similarities. The disaccharide spectinomycin is loosely regarded as an aminoglycoside but is truely an aminocyclitol antibiotic in that it lacks an aminosugar and differs functionally (Jacoby and Gorini, 1967; Rinehart, 1969; Benveniste and Davies, 1973 and Tanaka, 1975). Its relationship with streptomycin will be discussed later. Streptomycin contains streptidine, as shown in the first figure, is highly cationic by virtue of the twin guanido substitutions, is bacteriocidal and is most active around pH8. Originally isolated from S. griseus on the basis of its activity against the "tubercle bacillus", early applications were primarily in the treatment of tuberculosis. A broad spectrum antibiotic, it is used now to combat the bubonic plague, tularemia and brucellosis. This represents a limitation of its theoretical capabilities as a result of the ever-increasing incidence of clinically resistant bacteria and the simultaneous development of superior drugs (Schlessinger and Medoff, 1975). Human toxicity, mainly the dysfunction of the eighth cranial nerve, the etiology of which is not known, is another problem. This can arise from prolonged sustenance or elevated dose levels. The drug has little effect on anaerobic bacteria, fungi, protozoa or



	A	B	E
Streptomycin	CHO	H	CH_3
Hydroxystreptomycin	CHO	H	CH_2OH
Dihydrostreptomycin	CH_2OH	H	CH_3
Mannosidostreptomycin	CHO	↓	CH_3

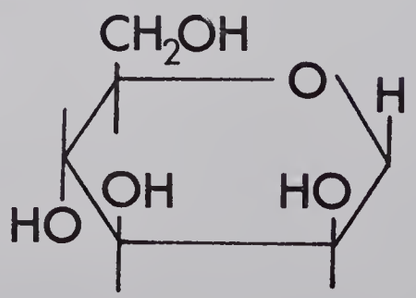


FIGURE 1: The structure of streptomycin and its derivatives. BLUENSOMYCIN is similar to dihydrostreptomycin except one of the guanido groups of streptidine is replaced with a urethan function ($-O-\overset{\overset{O}{\|}}{C}-NH_2$) (Horner, 1967 and Schlessinger and Medoff, 1975).

viruses (Weinstein, 1970)

Screening for biologically active derivatives, Davies et al (1969) discovered that only limited chemical modification of the molecule was possible. These derivatives are shown in the first figure as well. Mannosidostreptomycin, bluensomycin and hydroxystreptomycin are also products of Streptomyces, whereas dihydrostreptomycin is derived from the catalytic hydrogenation of streptomycin (Waksman 1953 and Schlessinger and Medoff, 1975). Dihydrostreptomycin and streptomycin reportedly have the same degree of antibacterial activity (Brock, 1966 and Harwood and Smith, 1969) but differ in their abilities to precipitate DNA in vitro (Brock, 1966), in degrees of human toxicity and in their efficacy against some Gram-negative bacteria (Schlessinger and Medoff, 1975). The loss of both of the guanido groups, hydrolysis or the blockage of the 3"OH of the N-methyl-L-glucosamine residue, or alkylation of the aldehyde groups will abolish activity.

The enzymatic modification of aminoglycosides can occur in nature. Enzymes capable of acetylation, adenylylation and phosphorylation, with the eventual inactivation of these drugs, have been described upon the isolation from bacteria (Benveniste and Davies, 1973). These are genetically inscribed on plasmids called resistance transfer factors (R factors). R factors, first identified by Japanese scientists in clinical isolates of Shigella (Akiba et al, 1960), are conjugally transferable amongst related Enterobacteriaceae and carry the determinants for multiple drug resistance. Bacteria harbouring R factors are thought to be primarily responsible for the increased incidence of antibiotic resistance in clinical isolates (Davies and Kass, 1971). Some Streptomyces species, essentially the aminoglycoside producers, have been shown to contain modifying enzymes as well (Benveniste and Davies, 1973a). Phosphotransferases and acetyltransferases

have been described. It has been hypothesized that this was the original source from which bacteria eventually aquired their enzymes (Davies and Benveniste, 1974). It is the particular interest of this report that certain bacteria possess the ability to adenylylate the sensitive 3"OH site of streptomycin (Takasawa et al, 1968; Umezawa et al, 1968; Yamada et al, 1968 and Harwood and Smith, 1969). These are clinically resistant bacteria. The same 3"OH is vulnerable to phosphorylation by bacteria that have the required enzyme (Ozanne et al, 1969 and Davies et al, 1971). The two processes are shown in figure 2 which highlights their similarities. A second form of adenylylation has recently been identified in S. aureus (Suzuki et al, 1975). Modification in this case has occurred at the C-6 locus of streptidine.

Biological inactivation of streptomycin was demonstrated initially in soluble extracts of a resistant E. coli carrying an R factor acquired from a naturally isolated Shigella species. Quantitating the streptomycin after periods of incubation in the inactivating mixture by assay against a sensitive organism reflected the progressive loss of activity through time (Okamoto and Suzuki, 1965). Yamada et al (1968), with a partially purified enzyme preparation from E. coli, isolated and characterized the inactivated product of streptomycin as being the 3"-adenylylated derivative as in the second figure. Activity could be restored to this preparation upon the incubation with snake venom phosphodiesterase indicating that inactivation was an addition reaction and distinguishing it from the phosphorylated derivative which can be reactivated with alkaline phosphatase. The reaction required ATP; no other nucleotide supported it. At the same time, another report (Umezawa et al, 1968) had indicated that ADP could replace ATP, however the crude enzyme preparation used in this work most likely generated ATP from the added ADP (Takasawa et al, 1968). The reaction

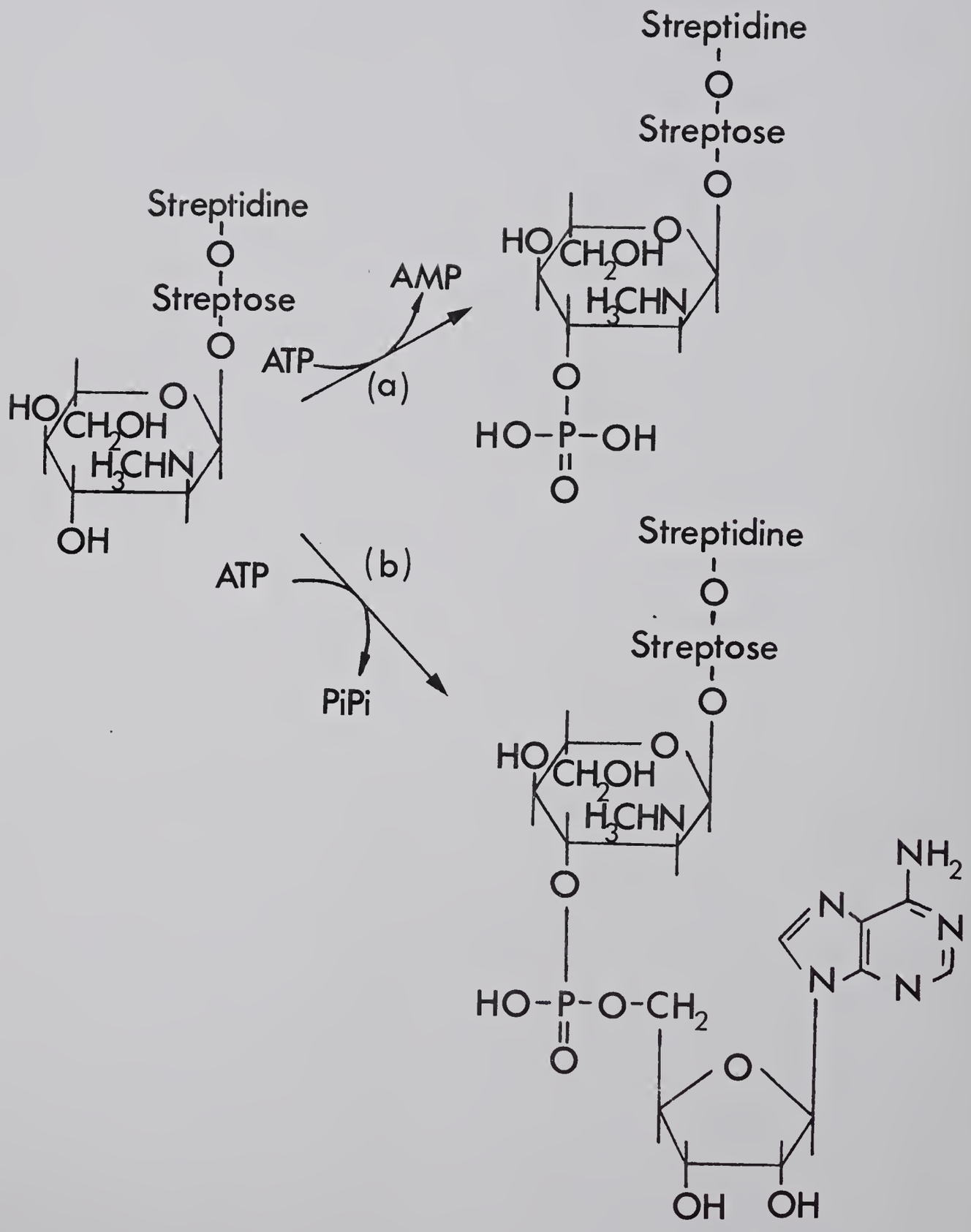

Streptidine
Streptose
Streptidine
Streptose
AMP
ATP
(a)
ATP
(b)
PiPi
Streptidine
Streptose

FIGURE 2: The enzymatic mechanisms of streptomycin inactivation, (a) phosphorylation and (b) adenylylation. Both occur at the 3"OH of the N-methyl-L-glucosamine residue (Franklin and Snow, 1971).

had a pH optimum around 8.5 at 37C and proceeded only in the presence of a divalent cation. Yamada's group included in their reaction mixture a univalent cation but this was later found to be stimulatory and not essential (Benveniste et al, 1960).

Derivatives of streptomycin can be adenylylated by the streptomycin 3"-adenylyltransferase (AAD-(3")) provided there is no modification at or near the 3"OH (Harwood and Smith, 1969). Mannosidostreptomycin is not inactivated (Davies et al, 1971). Spectinomycin (figure 3) is also inactivated by this enzyme. Benveniste et al (1970) screened fifteen different R factors known to carry streptomycin-resistant determinants in E. coli and discovered that eleven also coded for spectinomycin resistance. Adenylylation of the two by the same enzyme was confirmed chemically and genetically. The other four R factors coded for the phosphorylation of streptomycin and these R+ bacteria remained sensitive to spectinomycin. These same authors demonstrated a direct relationship between the degree of susceptibility of the R+ bacteria and the capacity of cell-free extracts to inactivate streptomycin.

AAD-(3"), despite attempts, has not been purified. Predictions of its molecular weight are around 3.3×10^4 daltons (Benveniste et al, 1970). In the absence of a divalent cation its activity can be irreversibly lost. It, along with other aminoglycoside inactivating enzymes, are constitutively produced (Yamada et al, 1968 and Harwood and Smith, 1969) and are quantitatively released from E. coli during cold-water shocking, when only 4% of the cellular protein is released (Yamada et al, 1968; Benveniste et al, 1970 and Davies and Benveniste, 1974). By definition this would classify them as periplasmic enzymes. A periplasmic location generally includes that area which is extracytoplasmic and cell-associated. This will include the cell wall, cell membrane, interstitial

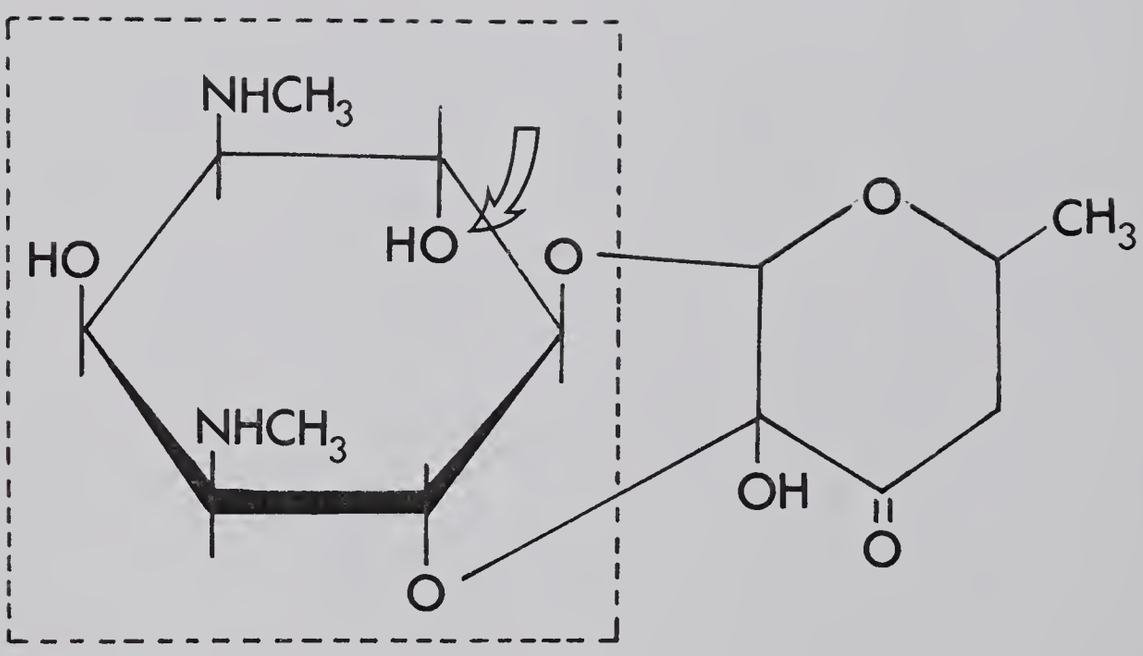

NHCH3
HO
HO
O
NHCH3
O
O
CH3
OH
O

FIGURE 3: The structure of spectinomycin. Outlined by the dotted box is actinamine, the substrate for the streptomycin-spectinomycin adenylyltransferase. The point of adenylylation is marked by the arrow (Benveniste and Davies, 1973).

spaces and even the internal face of the cytoplasmic membrane, anywhere from which protein can be released during the osmotic shocking procedure (Davies et al, 1971). Harwood and Smith reported a release of an adenylyltransferase of 50% during shocking, at which time 10% of the soluble protein beta-galactosidase was freed. Differences in release may be accountable on the basis of the age of the cells being shocked (Neu and Chou, 1967). Young, logarithmically growing cells tend to release less periplasmic enzyme than do stationary cells as were employed by Harwood and Smith and Benveniste et al, respectively. On the other hand, the differences may merely be reflective of the idiosyncrasies in technique. Sphaeroplasts of E. coli prepared by lysozyme and EDTA treatment retain inactivating enzymes while releasing the characteristic "free" periplasmic enzymes such as alkaline phosphatase. Lundbäck and Nordström (1974a) could release from E. coli sphaeroplasts 70% of the streptomycin adenylyltransferase but only very little of a streptomycin phosphotransferase which was coded for by another R factor suggesting that the two were periplasmic and cytoplasmic, respectively. In contrast, Ozanne et al (1969) had previously shown that a phosphotransferase could be released from shocked cells. Further, APH(3')-II, a phosphotransferase inactivating kanamycins A and B and butirosins (aminoglycosides), was accessible to specific antiserum in E. coli, therefore some fraction of it must lie outside the cell membrane (Matsuhashi et al, 1976 and 1976a). It is apparent that more study is warranted to clarify the present ambiguities concerning the location of these enzymes. Localization of the periplasmic proteins has long been known to be a troublesome problem (Heppel, 1971).

The emergence of resistance to streptomycin followed shortly after the application of the drug clinically. By 1946 clinically resistant bacteria

had proven to be a major therapeutic problem (Klein and Kimmelman, 1946). Clinically isolated Mycobacterium had been recorded as being resistant (Youmans and Feldman, 1946) as had been several other bacterial species (Buggs et al, 1946). Resistant laboratory isolates, selected from sensitive parents, were widely reported (Klein and Kimmelman, 1946; Miller and Bohnhoff, 1946 and Newcombe and Hawirko, 1949). Most of the lab isolates demonstrated a high degree of tolerance, up to 4,000ug/ml in E. coli, for streptomycin (Newcombe and Hawirko, 1949). Moderate to low level forms of tolerance, analogous in degree only to R factor-mediated resistance, were observed at the same time. In Shigella, at least, the highly resistant forms remained sensitive to low doses of penicillin and sulphonamides (Klein and Kimmelman, 1946). Drug inactivation was apparently not a factor in the resistance of the high level type in E. coli (Seligman and Wasserman, 1947). Isolated from a population of resistant cells was a third phenotype, dependence, described in E. coli where some cells had developed a nutritional requirement for streptomycin (Newcombe and Hawirko, 1949). Later, from crosses in the same organism, the three phenotypes could be shown to be determined by a single gene locus (Newcombe and Nyholm, 1950). Presumably these were all mediated by an alteration affecting a cellular target site of the drug, as had been proposed in the theoretical arguments of Sevag (1946).

The first publications of work directed at identifying the effects of streptomycin on bacteria and, by inference, the target site, reported alterations seen with the cells oxidative processes. Using susceptible populations of resting cells, benzoic acid oxidation was inhibited by streptomycin in Mycobacterium (Fitzgerald and Bernheim, 1947), in E. coli amino acid oxidation was inhibited (Geiger, 1947) as was the oxidation

of selected carbohydrates in Shigella sonnei, S. aureus and B. cereus (Henry et al, 1948). In this third report indications were that purified enzyme systems, normally involved in oxidative pathways were unaffected by streptomycin in vitro. Resistant bacteria failed to display these effects. Umbreit (1949) and then Oginsky et al (1949) suggested that a block in terminal respiration was responsible for the phenomena observed to date, most likely at or near the point of the pyruvate-oxaloacetate condensation. Reduced oxygen consumption was detectable as well in populations of growing cells (Henry et al, 1949)

A failure to consistently demonstrate a direct relationship between streptomycin action and reductions in oxygen consumption cast doubts on the primary involvement of terminal respiration (Paine and Clark, 1954). More significant was the relationship that existed between oxygen consumption or active metabolism in the absence of streptomycin and sensitivity to the drug (Rosenblum and Bryson, 1953; Paine and Clark, 1954 and Hurwitz et al, 1955). Anaerobiosis, low temperatures and chloramphenicol treatments all antagonized the action of streptomycin in S. aureus (Hancock, 1960). In addition, resting cells were considered less sensitive (Hurwitz et al, 1955 and Hancock, 1960). This led Hancock to suggest that it was the access to or the action at the target site which was dependent on an aerobic process. Respiratory enzymes were thought to be the streptomycin sensitive elements (Hancock, 1960 and 1960a). Interference of these by streptomycin was felt to explain the general inhibition of macromolecular syntheses he had seen during the in vivo action of the drug. Considering this possibility, Michalski (1959) assessed the sensivity of some isolated respiratory-linked dehydrogenases. He found inhibition so low as to preclude any significance to the in vivo effects.

Two other hypotheses were advanced at this time regarding possible mechanisms of action. Rosenblum and Bryson (1953) conjectured an inhibition of nitrogen assimilation. Hurwitz and Rosano (1958) followed by suggesting that it was a component of phosphate metabolism which was being inhibited. The former was considered because under conditions of nitrogen limitation the addition of exogenous glucose in the defined medium failed to enhance the streptomycin action. The fact that phosphate supplements to the growth medium antagonized the drug prompted the latter proposal.

The concern of the investigators in this area was to distinguish between the primary and subsequent effects induced by streptomycin. Hancock (1961) argued that the reductions seen in oxidative activity in E. coli and B. megaterium, which were concommitant with growth inhibition, were not secondary to the membrane damage witnessed by Anand and Davis (1960) in E. coli for he failed to detect any general breakdown of this barrier in his bacteria. He contested that the effect on oxidation by streptomycin is not a simple consequence of growth inhibition as other antibacterial agents fail in inducing the same response (Hancock, 1960a).

Implications of the bacterial membrane as a possible target site followed the discoveries of Anand and Davis (1960). Shortly after the addition of streptomycin, E. coli cells leaked small molecules and 5'-nucleotides indicative of degenerative changes to the membrane permeability barrier. The nucleotide excretion was verified in several reports and proven to involve nucleotides from de novo synthesis (Rosano et al, 1960 and Roth et al, 1960). Anand and Davis also detected an inhibition of protein synthesis and later, of nucleic acid synthesis. Early competence of the protein synthetic machinery however, was considered necessary for streptomycin activity. Chloramphenicol abolished activity

as had been seen before and the starving of an amino acid auxotroph was protective. A later study challenged the second observation with data showing that starved amino acid auxotrophs, whose protein synthesis was 0.5% of normal, remained sensitive (Stern et al, 1966). Anand and Davis concluded that streptomycin acted to disturb the membrane-forming apparatus of growing cells leading to improper assembly and subsequent leakiness. The dominance of sensivity over resistance, described by Lederberg in 1951, could be rationalized on this basis whereas a routine explanation for this observation was lacking in the argument that streptomycin inhibits an participating in a metabolic pathway.

A better understanding of the overall mechanism of resistance was offered by Szybalski and Mashima (1959) who had compared the levels of irreversibly bound ^{14}C-streptomycin in sensitive, dependent and resistant variants of an E. coli strain. Sensitive cells rapidly bound and eventually succumbed to 10^5 to 10^6 molecules per cell, figures which were duplicated later by Hurwitz and Rosano (1962). This represented concentrations a hundred times greater within the cells than in the surrounding medium. Resistant bacteria failed to accumulate drug and dependent cells showed only a low, transient amount of bound label through the same time period. Surveys, since this work, have indicated in E. coli (Carlson and Bockrath, 1970 and Lundback and Nordstrom, 1974a), in Streptococcus (Perry, 1969), in P. aeruginosa (Tseng et al, 1972 and Bryan et al, 1975) and in S. aureus (Hancock, 1962) that a direct correlation exists between susceptibility and the capacity of an organism to accumulate streptomycin.

The basis of resistance and susceptibility to streptomycin was hypothesized by Spotts and Stanier (1961) to implicate cellular ribosomes based on the epiphenomena characteristic of dependent bacteria.

Flaks et al (1962 and 1962a) proceeded to show that the drug could inhibit protein synthesis in vitro using synthetic messenger and that the ribosomes were the sensitive element. The 30S ribosomal subunit from susceptible bacteria bound streptomycin tightly at a one-to-one ratio (Chang and Flaks, 1972). Mutations to resistance and dependence involved a change in the single polypeptide of 30S subunits, S12 (P10) (Cox et al, 1964 and Ozaki et al, 1969). In vitro protein synthesis using ribosomes from resistant cells was not inhibited nor did these structures effectively bind streptomycin. Binding by ribosomes from dependent cells was only slightly better (Davies, 1964). Miscoding, the substitution of isoleucine for phenylalanine during translation of polyU messenger, can be induced with streptomycin-treated sensitive ribosomes but not resistant ones (Davies et al, 1964). The phenomenon of miscoding is evident in vivo as phenotypic repair, the ability of streptomycin to suppress missense mutations (Gorini and Kataja, 1964). This particular attribute supported the theory introduced by Hurwitz and Rosano (1962) that "faulty" protein induced by streptomycin as a result of miscoding was being incorporated into growing membrane causing the observed changes in its integrity. Modolell and Davis (1969) predicted that these effects on the ribosome brought about by the binding of the drug were a result of a conformational alteration in the A site, or recognition site.

Another form of resistance has been described recently. Certain bacteria can tolerate normally lethal doses of streptomycin while not possessing inactivating enzymes or resistant ribosomes. Apparently they are less permeable to the drug for reasons associated with the active uptake mechanisms, as in P. aeruginosa (Bryan et al, 1975 and Kono and O'Hara, 1975), and possibly S. typhimurium (Yamada and Davies, 1971), or because of what appears to be an impermeable cell wall such as in

Enterococcus (Moellering et al, 1970). In at least one example the reduced permeability is allegedly R factor-mediated (Kono and O'Hara, 1975).

An awareness of the method by which streptomycin enters a bacterial cell has become essential to the discussion of susceptibility. The status of the intracellular site has been shown not to be the sole determining factor in susceptibility. In recent years the pattern and mechanics of streptomycin uptake have received considerable attention. Upon exposure to the drug a rapid binding to cells is evident (Szybalski and Mashima, 1959 and Anand et al, 1960). This attachment is reversible (Hancock, 1962 and McQuillen, 1951), independent of temperature (Anand et al, 1960), unaffected by metabolic inhibitors (Hancock, 1962; Hurwitz and Rosano, 1962 and Bryan and Van Den Elzen, 1976), concentration dependent (Hurwitz and Rosano, 1962) and dependent on the ionic constitution of the medium (Beggs and Andrews, 1976 and Plotz et al, 1961). Consequently, this is considered to be surface bound drug maintained through ionic forces. The ability of streptomycin to reduce the net negative surface charge and hence a cells electrophoretic mobility is corroborative with this conclusion (McQuillen, 1951).

Subsequent uptake can be divided into two stages. The first, by reports of work with E. coli, represented a lag phase during which no further uptake was visible (Anand et al, 1960 and Plotz et al, 1961). Some investigators failed to detect this phase completely such as Szybalski and Mashima (1959) and Hancock (1962 and 1962a), the latter of whom worked with B. megaterium. More recently in E. coli, Bryan and Van Den Elzen (1976) have shown that accumulation continues but at low rates. Differences in observations may be due to the fact that the duration and the rate of this phase is concentration dependent (Hurwitz

et al, 1962 and Bryan and Van Den Elzen, 1976). Uptake in this phase can be antagonized by divalent cations in whole cells and sphaeroplasts of E. coli (Bryan and Van Den Elzen, 1977), inhibited by the electron transport chain antagonists cyanide, azide and anaerobiosis and can be reduced with the lowering of the temperature (Bryan and Van Den Elzen, 1976). The sulphydryl group reactant N-ethylmaleimide (NEM) was shown to abolish uptake in this phase as well. This explains the protective nature of iodoacetate (Hurwitz and Rosano, 1958) and the other inhibitors mentioned earlier. Chloramphenicol only reduced uptake by a third through this phase (Bryan and Van Den Elzen, 1976). The earliest effect on cells can be seen during this period. Potassium efflux has been witnessed in E. coli (Dubin et al, 1963) and in B. megaterium (Hancock, 1964).

The third phase of uptake is more rapid than the second but is sensitive to the same inhibitors even when added, in some cases, after the onset of this phase (Hancock, 1962; Andry and Bockrath, 1974; Bryan and Van Den Elzen, 1976 and Beggs and Andrews, 1976), a time when membrane degeneration is reportedly occurring (Dubin et al, 1963). Magnesium and calcium ions reduce third phase uptake in E. coli sphaeroplasts (Bryan and Van Den Elzen, 1977) and phosphate has been shown to direct its antagonistic effect here also, except in this case, with whole cells (Hurwitz, 1962a). Brock (1966) stated that phosphates interfere with streptomycin directly by electrostatic interactions. Inhibition of this phase with chloramphenicol is complete if it is added prior to its onset; its effects after this time diminish relative to the length of the delay (Hurwitz, 1962a). In all the reports referred to where uptake phenomena had been studied, it was the third phase of uptake that was absent in resistant bacteria. As a result, initiation of the

third phase would appear to be linked to the binding of drug to the ribosome. Several other facts bear this out. Chloramphenicol is known to stabilize polyribosomes, a state in which the ribosomes are not effective in binding streptomycin. Puromycin, which acts to disrupt polyribosomes, is synergistic with streptomycin (Brock, 1966). This too would explain the effect of chloramphenicol without implicating the direct involvement of protein synthesis. Simultaneous with the beginning of the third phase of uptake is the inhibition of protein synthesis and the loss of viability (Hurwitz and Rosano, 1962; Dubin et al, 1963; Hancock, 1964 and Bryan and Van Den Elzen, 1977). Dubin et al (1963) noted that the gross permeability changes, the inhibition of nucleic acid synthesis and the drop in oxygen consumption are detectable later.

The second and third phases of uptake are energy-dependent (Andry and Bockrath, 1974 and Bryan and Van Den Elzen, 1976) contrary to Anand et al (1960) and Dubin et al (1963) who envisioned the passive diffusion through compromised membranes. Due to the inhibition by anaerobiosis, the source of the required energy is considered to be linked to aerobically driven electron transport. Anaerobically generated ATP seems ineffective in supporting transport (Bryan and Van Den Elzen, 1976). These authors argue that the energy is used solely for the transport of drug for several reasons. Energy has not been shown to be required for the binding of streptomycin to ribosomes. Resistant bacteria require energy to transport drug during the second phase and this is in the absence of any binding to ribosomes. Anaerobically, ribosomes would be expected to remain fully competent to bind drug. Kogut et al (1965) have demonstrated that dihydrostreptomycin will kill E. coli in the absence of oxygen so long as uptake occurs aerobically.

The cell wall peptidoglycan layer appears to participate in a penetration barrier to aminoglycosides. Carbenicillin acted synergistically with streptomycin against P. aeruginosa (Bryan et al, 1975), at the same time as promoting the uptake of the drug. Synergism between penicillin and streptomycin against Streptococcus has been known for some time (Moellering et al, 1970). The uptake of streptomycin in Enterococcus is similarly enhanced by penicillin treatment (Zimmerman et al, 1971).

Bryan and Van Den Elzen (1977) suggested that membrane energy complexes involving respiratory quinones behave as agents, or react stoichiometrically with the elements, that bind and transport aminoglycosides. Quinone deficient mutants of E. coli are less sensitive to streptomycin and transport can be saturated in these mutants at drug concentrations below that required by the parent strains.

It is debatable whether streptomycin is concentrated internally in an unbound form as active transport is partly defined. Sensitive cells can accumulate drug to internal concentrations amounting to 300 to 400 times the external concentrations with the associated drug being unmodified (Bryan and Van Den Elzen, 1976). Earlier reports confirm the absence of modification upon internalization (Hancock, 1962a and Hurwitz and Rosano, 1962). The drug would be expected to bind readily to anionic components of the cell such as seen with nucleic acids (Cohen, 1947). Hurwitz and Rosano (1962) and Andry and Bockrath (1974) observed that streptomycin and dihydrostreptomycin respectively, having been taken up by sensitive E. coli could be partially released during toluene disruption of the membrane permeability barrier. However, it is uncertain whether this represents the release of free drug or possibly drug associated with toluene-sensitive receptors.

Adenylylstreptomycin is not bacteriocidal. In addition it does not inhibit protein synthesis, induce miscoding or support the growth of dependent bacteria. Binding to ribosomes is very weak, being readily displaceable by active drug if it binds at all (Yamada et al, 1968). Typically, R factor strains accumulate less drug than their R- parents, in P. aeruginosa (Bryan and Van Den Elzen, 1975 and Bryan et al, 1975) and in E. coli (Lundbäck and Nordström, 1974). Judging from the earlier observations it would be expected that these bacteria lack, at least, the third phase of uptake. Resistance might be explained on the basis of the drugs inability to reach the ribosomal site or its inability to bind once there.

This project has attempted to study the effects on the uptake of dihydrostreptomycin by E. coli which possess R factor-mediated streptomycin adenylylating activity. It also includes attempts to identify and to localize drug that has been accumulated by these strains. The cellular localization of the modifying enzyme is another aspect that has been approached. The immediate goal was to correlate the findings to further clarify the mechanism by which bacteria with aminoglycoside-inactivating enzymes are resistant. Ultimately it is hoped that this work will have been a progression in the direction of a better understanding of aminoglycoside resistance, transport and lethality in bacteria; if so, then we will be closer to the realization of the ideals outlined in the opening paragraphs.

MATERIALS AND METHODS

1. Chemicals and reagents.

All chemicals used were of reagent grade and were acquired from commercial suppliers. Obtained from the Sigma Chemical Company were the following: methanesulphonic acid ethyl ester (EMS), p-nitrophenyl-phosphate(disodium) (pNPP), beta-nicotinamide adenine dinucleotide, reduced form (NADH), lysozyme (25,000u/mg), deoxyribonuclease I (DNase) (2,010u/mg) and trypsin inhibitor (1mg inhibits 1.5mg of trypsin of approximately 10,000 BAEEu/mg). Trypsin (9,000 BAEEu/mg) was obtained from Miles-Seravac Ltd.; ribonuclease A (RNase) (5,100u/mg) from Worthington Biochemical Corp.; (adenine-U-^{14}C)adenosine-5'-triphosphate(ammonium salt) (268mCi/mmol) from Amersham; 4-acetamino-4'-isothiocyanotostilbene-2,2'-disulphonic acid(sodium salt) (SITS) from Serva Feinbiochemica, Heidelberg; Triton X-100 from Beckman Instruments Inc.; di-2-ethyl hexyl phosphate from Koch-Light Laboratories, England; o-nitrophenyl-beta-galactopyranoside (ONPG); p-toluene-sulphonic acid monohydrate from Terochem Laboratories, Edmonton and Omnifluor from New England Nuclear.

2. Antibiotics.

Dihydrostreptomycin sulphate (DHS) was obtained from Sigma; ^{3}H-dihydrostreptomycin sesquisulphate (3Ci/mmol) from Amersham; spectinomycin (trobocin) from Upjohn Co. and sensitivity discs were procured from Baltimore Biological Laboratories (BBL).

3. Buffers.

(i) Standard buffer: 10mM Tris-chloride pH7.8, 30mM NaCl.

(ii) Buffer A: 10mM Tris-chloride pH8.3, 10mM $MgCl_2$, 1.0mM dithiothreitol.

4. Media.

Growth media used are listed below along with the name of the supplier.

Nutrient broth (NB) (BBL)

Trypticase soy broth (TSB) and agar (TSA) (BBL)

Mueller-Hinton agar (BBL)

Minimal salts (per litre)

10.5g K_2HPO_4

4.5g KH_2PO_4

1.0g $(NH_4)_2SO_4$

0.5g sodium citrate.$2H_2O$

1ml 1M $MgSO_4.7H_2O$ (added after autoclaving)

Minimal medium, in addition to the minimal salts, included 10ml of a 20% solution of glucose and 0.5ml of 1% thiamine hydrochloride. 15g of Difco agar was included in the preparation of minimal agar plates.

5. Bacterial strains.

The following is a list of the bacteria employed throughout this study and their sources.

(i) E. coli K12 J5 (F^- lac^+ pro^- met^- $Azide^r$) R71-a from Y. Chabbert, Pastuer Institute, Paris, France.

(ii) E. coli K12 SA1306 (pro^- met^- Nal^r) from K. Sanderson, Univ. of Calgary, Calgary, Alberta.

(iii) E. coli K12 SA1306 (pro^- met^- Nal^r Rif^r) by selection for resistance to 100ug/ml rifampicin in TSA.

(iv) E. coli K12 SA1306 (pro^- met^- Nal^r Rif^r Str^r) by selection for resistance to streptomycin at 2,000ug/ml in TSA.

(v) E. coli ML308.225 (i^- z^- a^+ y^+) from M. Pickard, Univ. of Alberta, Edmonton, Alberta.

(vi) E. coli ML308.225 (i^- z^- a^+ y^+ Rif^r) by selection for resistance to 100ug/ml rifampicin in TSA.

(vii) E. coli ML308.225 (i^- z^- a^+ y^+ Rif^r Str^r) by selection for streptomycin resistance at 250ug/ml in TSA.

(viii) E. coli K12 Strain 7 (Hfr) from B. P. Rosen, Univ. of Maryland, Baltimore, Maryland.

(ix) E. coli K12 Strain 7 (Hfr Rif^r) by selection for resistance to 100ug/ml rifampicin in TSA.

(x) E. coli K12 NR-70 (Hfr $ATPase^-$ Unc^-) from B. P. Rosen, Univ. of Maryland, Baltimore, Maryland.

(xi) E. coli K12 NR-70 (Hfr $ATPase^-$ Unc^- Rif^r) by selection for resistance to 100ug/ml rifampicin in TSA.

6. Disc sensitivity tests.

Antibiotic sensitivity profiles were assessed according to the method reported by Bauer et al (1966) on Mueller-Hinton agar.

7. Minimum inhibitory concentration (MIC) determinations.

A conventional tube dilution procedure was applied to determine antibiotic susceptibilities. Bacterial suspensions, diluted from logarithmically growing cultures, were mixed with two-fold dilutions of antibiotic in NB (unless indicated otherwise) such that the final concentration of bacteria was 10^5 cells per millilitre in a volume of 1ml (in 16x100mm tubes). The last tube demonstrating growth upon visual inspection after 18 hours incubation at 37C was regarded as the MIC.

8. Mating systems.

Matings were accomplished by employing one of the two systems described below.

Liquid matings: Donor and recipient strains were grown in TSB to mid-to-late logarithmic phase then adjusted to an OD_{600} of 0.5 (Spec-

tronic 20, Bausch and Lomb). One millilitre of donor was mixed with 9 ml of recipient in a 150ml medicine bottle. The bottle was incubated, flat side down, at 37C for 90 minutes after which aliquots of several ten-fold dilutions of the mating mixture were spread on selective media.

Filter matings: Donor and recipient strains were mixed as above then 2 to 3ml was impinged onto 47mm x 0.2u membrane filters (Sartorius), washed with 20ml of isotonic saline and the filters overlayed on pre-warmed TSA plates. The plates were incubated overnight at 37C. The following day growth was resuspended in TSB, serially diluted and aliquots were spread on selective plates. The procedure is a modification of that reported by Matney and Achenbach (1962).

Selection for transconjugants (bacteria having received the R factor by conjugation) was on TSA supplemented with 100ug/ml and 12.5ug/ml of rifampicin and dihydrostreptomycin, respectively, except for those crosses involving E. coli strains 7 and NR-70 in which cases the concentration of dihydrostreptomycin was 50ug/ml.

9. R factor stability.

Chandler and Krishnapillai originally reported this methodology in 1974. Strains were grown overnight in TSB with 15ug/ml DHS. The following day aliquots were subcultured, to 100cells/ml, in drug-free TSB and grown for 8-9 hours at 37C, thus allowing for 10 to 15 generations of growth. Dilutions of both the initial and the final cultures were spread on TSA plates and grown overnight at 37C. Plates with isolated colonies were replica plated, using a velvet-topped replicating block, onto TSA with 15ug/ml DHS to determine the percentage of clones that retained the R factor. At least 100 clones were plated in order to determine the percentages.

10. Mutagenesis of E. coli K12 SA1306 R71-a.

Mutagenesis was carried out using EMS according to the method of Osborn et al (1967). Cells were grown in TSB to 0.5 OD_{600} units/ml then concentrated two-fold by filtration (47mm x 0.2u membrane filters, Sartorius) and resuspension into minimal salts. Two millilitres of this plus 7.6ml of 1M Tris-chloride pH7.4 and 0.4ml of EMS were combined and swirled vigorously for 5 minutes at 37C. The culture was then serially diluted and aliquots spread on TSA plates which were incubated overnight at 37C. Next day, colonies were patched, in replica, using sterile toothpicks to TSA supplemented with 100ug/ml rifampicin with or without 12.5ug/ml of DHS. Those clones showing reduced growth on the DHS supplemented plates were selected and isolated for future study.

11. Adenylyltransferase assays in cell-free extracts.

To assess the capacity of an organism to inactivate DHS by adenylylation, assays were performed using either osmotically shocked or sonicated cell-free extracts.

Osmotic shocking: The following procedure was reproduced from Benveniste et al (1970). Cells were harvested by centrifugation from TSB at 0.7 to 0.8 OD_{600} units/ml and washed twice in twelve volumes of standard buffer. The washed cells were resuspended into 36 volumes of 0.033M Tris-chloride pH7.8, 20% sucrose and $3x10^{-3}$M EDTA and stirred at room temperature for 20 minutes. After pelleting at 16,000xg for 15 minutes, the cells were then resuspended into 24 volumes of cold $5x10^{-4}$M $MgCl_2$ and stirred vigorously for 20 minutes at 4C. Stirring was accomplished through the use of a magnetic stirring rod. The supernatant of a subsequent 30 minute, 26,000xg centrifugation at 4C was the osmotic shock fluid.

Sonication: Cells were harvested and washed as above. Resuspension was into a small volume of buffer A in 16.1mm x 76.2mm clear polycar-

bonate tubes. Sonication of this suspension was carried out for 2 minutes on ice, in four 30 second intervals at a setting of 55 on a Biosonik III (Bronwill Scientific Inc.) cooling the probe for one minute between blasts. The supernatant of a 30 minute, 40,000xg centrifugation represented the cell-free sonic extract.

The extracts were assayed for adenylyltransferase activity using the following methodology as fashioned after Ozanne et al (1969).

The reaction mixture (per 70ul) contained:

3 umoles Tris-chloride pH8.3

0.6 umoles $MgCl_2$

70 nmoles dithiothreitol

10 nmoles DHS

60 nmoles ^{14}C-ATP (2uCi/umole)

20ul enzyme preparation

Reactions proceeded for the desired length of time at 30C. Aliquots (10 or 20ul) were taken and spotted onto 0.75cm^2 pieces of phosphocellulose paper (Whatman P-81) which were allowed to air-dry for ten seconds prior to being immersed in distilled water at 90C for two minutes to terminate the reaction. The papers were washed three times in large volumes of distilled water, dried at 80C then counted using a toluene based scintillation fluid (Omnifluor 4g/litre) in a Beckman model LS-250 scintillation counter. Controls lacked either the enzyme or DHS. For the measurement of initial velocities assay volumes were halved and ATP concentrations were 1.5mM. Reactions were terminated rapidly by heating the assay mixtures to 90C for one minute. One enzyme unit (EU) of activity produced lumole of product per hour.

Protein was determined by a modified Lowry method described by Hartree (1972).

12. Marker enzyme assays.

Spectrophotometric analysis of reaction products was accomplished using a double-beam Unicam SP-1800.

Beta-galactosidase: The reaction mixture included:

0.9ml Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4.7H_2O$ and 50mM mercaptoethanol) pH7.0

0.2ml ONPG (4mg/ml)

0.1ml enzyme preparation

The mixture was incubated for the desired length of time (1-2 hours) at 30C then terminated with the addition of 0.5ml of 1M Na_2CO_3. The A_{420} was determined. 1EU of activity produced one umole of product per hour. Controls lacked the enzyme. The methodology was taken from Miller (1974).

Alkaline phosphatase: Reaction mixtures included:

0.85ml 1.0M Tris-chloride pH8.0

0.05ml pNPP (4mg/ml)

0.1ml enzyme preparation

Incubation was at 30C for the duration (1 to 2 hours) at which time the reaction was stopped with 4ml of 0.1M Na_3PO_4 and the A_{420} determined. 1EU of activity produced one umole of product per hour. Controls lacked the enzyme.

Lactate dehydrogenase: Included in the reaction mixture were:

0.85ml 0.05M sodium phosphate buffer pH7.6

0.05ml 3mM NADH

0.05ml 0.5M sodium pyruvate pH7

0.05ml enzyme preparation

Ten seconds after mixing the reagents the A_{340} was recorded at 15

second intervals at room temperature in order to determine initial velocities. One EU of activity oxidized one umole of NADH per minute. Controls lacked pyruvate.

Uridine phosphorylase: The assay was adapted from Pardee and Watanabe (1968). Reaction mixtures included:

0.85ml 0.1M potassium phosphate buffer pH7.1

0.1ml 10mM uridine

0.05ml enzyme preparation

The above was mixed and incubated at 37C for 30 minutes then 0.3ml of cold perchloric acid was added. The precipitate was cleared by centrifugation and 0.2ml of supernatant was added to 0.8ml of 0.1M NaOH. The A_{295} of this solution was determined. One EU of activity produced an A_{295} change of 0.001 per second. Controls lacked uridine.

Dry weight determinations: Enzyme activities are primarily expressed per gram dry weight of cells. Dry weights were calculated by the following method. Cells were grown to mid-log phase in TSB. Ten millilitre aliquots were filtered through preweighed 47mm x 0.2u Sartorius membrane filters, washed with 20ml of isotonic saline then dried to a constant weight at 150C.

13. Ribosomal binding of dihydrostreptomycin.

Ribosomes from selected strains were prepared following the method described by Modolell (1971). The assay for the binding of DHS to the ribosomes has been described by Chang and Flaks (1972).

Preparation of crude ribosomal fractions: Cells were grown to approximately 0.5 OD_{600} units/ml in TSB and washed once with 10mM Tris-chloride pH7.8, 6mM 2-mercaptoethanol, 60mM NH_4Cl and 10mM magnesium acetate then frozen in liquid nitrogen and maintained at -70C until used. The frozen cells were ground with 1.5X their weight in levigated

alumina with a mortar and pestle. The ensuing paste was suspended in the above buffer, 2 volumes per gram wet weight of cells, added in three portions. The suspension was centrifuged at 20,000xg for 15 minutes, the supernatant decanted and DNase added to a final concentration of 3ug/ml. This was subsequently centrifuged at 4C for 35 minutes at 30,000xg. The supernatant was aspirated, dialyzed versus 500 volumes of buffer for 4-5 hours then centrifuged at 30,000xg for 20 minutes.

The S-30 supernatant, so prepared, was spun at 160,000xg for one hour (Beckman model L2-65B ultracentrifuge). The pellet was washed three times in assay buffer, 50mM Tris-chloride pH7.8, 50mM KCL and 10mM magnesium acetate. Resuspension in each case was promoted by gentle working of the pellet with a blunt-ended glass rod. Washed ribosomes were then dialyzed overnight against 500 volumes of the assay buffer at 4C, frozen in liquid nitrogen and maintained at -70C until used. Ribosomal preparations were never refrozen.

Binding assay: Ribosomes were diluted to 4.0×10^{-7}M assuming that 66ug of ribosomes equalled 24pmoles and that the concentration of ribosomes in mg/ml was given by the equation $(A_{260} \times 500)0.04$ (Modolell, 1971). ^{3}H-DHS was made to 3.5×10^{-6}M (sp. act. 50cpm/ng). Equilibrium dialysis was carried out in single-cell dialysis chambers (CRC, The Chemical Rubber Co.) with 0.4ml of the ribosomal preparation and 0.4ml of ^{3}H-DHS on either side of a dialysis tubing membrane (Fisher Scientific) washed according to Chang and Flaks (1972). Mixing was aided in individual cells by the use of glass beads while the chambers were rocked at room temperature. Samples (20ul) were taken, spotted on prewashed (with ribosomal diluent) nitrocellulose filters (Sartorius), dried at 80C and counted. Readings were made until equilibrium was established.

14. Sphaeroplast preparation.

Sphaeroplasts were formed using a modified version of a method published by Weiss (1976). Cells were grown to mid-log phase (0.3-0.6 OD_{600} or 0.5-0.9 OD_{450}) in minimal media and harvested by centrifugation. Following two washes with standard buffer the cells were resuspended into prewarmed (37C) 0.1M Tris-chloride pH8 and 20% sucrose in a volume equal to one-tenth the product of the initial cultures volume in millilitres and its OD_{450}. The suspension was transfered to an erlenmeyer flask and stirred gently using a magnetic stirring rod while maintaining the temperature at 37C. After 15 minutes lysozyme (2mg/ml) was added to a concentration of 100ug/ml. Twelve minutes later EDTA (0.1M NaEDTA pH8) was slowly added dropwise to a final concentration of 10mM while the culture was continuously stirred at 37C. When at least 99% of the cells had assumed a spherical shape, judging by examination under phase contrast microscopy, the suspension was sedimented at 3,600xg for 20 minutes at 4C and the cells resuspended into 10ml (per 400ml original culture) of 5mM Tris-chloride pH8 and 20% sucrose. DNase and RNase and Mg^{++} were added to 100ug/ml, 30ug/ml and 10^{-3}M, respectively, and the mixture incubated for 30 minutes at 37C, gently swirling for the duration.

To test for the osmotic fragility of the preparations small aliquots were diluted one-to-ten into 5mM Tris-chloride and 20% sucrose and into distilled water. A difference in OD_{600} between the two of ten-to-one was regarded as near optimal and only these sphaeroplasts were used for further study.

The pelleting of sphaeroplasts was always at 3,600xg for 20 minutes at 4C. Resuspension was facilitated by the use of a pasteur pipette. Sphaeroplasts were always used immediately.

15. Accumulation of dihydrostreptomycin in whole cells and sphaeroplasts.

Cell-associated drug was followed through time after the introduction of ^{3}H-DHS to growing cultures. The methods are essentially those of Bryan and Van Den Elzen (1976).

In whole cells: Uptakes were performed in 10ml of NB in 125ml erlenmeyer flasks at 37C in a New Brunswick reciprocal water bath shaker. ^{3}H-DHS (2-60cpm/ng) was added in a small volume to log-phase cells between 0.25 and 0.5 OD_{600} units/ml to the desired concentration at zero time. At specified times thereafter 1.2ml aliquots were removed from the incubating mixture, OD_{600} recorded, and then filtered through 25mm x 0.2u membrane filters (Sartorius) that had been prewashed with 2.5ml of DHS (2,500ug/ml). The filtered cells were washed with 20ml of 3% saline, dried at 80C and counted as before. Cultures pretreated with potassium cyanide (KCN) were done so under normal incubation conditions 5 minutes prior to zero time at a concentration of 1mM.

In sphaeroplasts: Sphaeroplasts were resuspended into prewarmed NB with 20% sucrose to approximately 0.5 OD_{600} units/ml and incubated for 15 minutes in the water bath prior to zero time. The uptake study proceeded from this point as in whole cells.

16. Detection of inactivated drug in the growth medium and in the cell.

The fate of inactivated drug was followed by the paper chromatographic analysis of extracts from growing cells as described below.

Extracellular inactivation: Logarithmically growing cells were diluted into TSB to 0.1 OD_{600} units/ml and ^{3}H-DHS (100cpm/ng) added to 50ug/ml. The culture was incubated for four hours then filtered through 47mm x 0.2u membrane filters (Sartorius). Small aliquots of the culture filtrates were chromatographed by a method described by

Wagman and Weinstein (PC method #1) (1973). The chromatogram was developed with water-saturated n-butanol with 2% toluenesulphonic acid in an ascending fashion for a period of 48 hours. The paper used was Whatman #1. Upon completion the paper was dried at 80C, cut into 1cm fractions and counted. Controls included the filtrate of a culture of strA bacteria and ^{3}H-AMP-DHS (adenylylated-DHS) obtained from the adenylylation of ^{3}H-DHS by cell-free extracts. Adenylylated drug was diluted 1:1 in double-strength TSB prior to being chromatographed.

Intracellular inactivation: Cells were grown to mid log-phase (0.3 OD_{600} units/ml) in 100ml of NB. To duplicate cultures was added ^{3}H-DHS (100cpm/ng) to a concentration of 1.0ug/ml. After periods of 15 and 30 minutes one culture was rapidly chilled on ice and a small volume extracted for the determination of cell-associated label as per an uptake measurement. The remainder of the culture was centrifuged at 4C at 12,000xg for ten minutes and washed twice with 3% NaCl. The washed pellets were then resuspended into 1.0ml of buffer A and sonicated as previously described. Aliquots of the supernatant of a 30 minute, 40,000xg centrifugation were chromatographed as per the PC method #3 of Wagman and Weinstein (1973). Development was with the organic phase of a 1:1 mixture of amyl alcohol with 1% di-2-ethyl hexyl phosphate and 0.1M borate buffer with 0.5% NaCl, the whole of which was adjusted to pH8 with NaOH. The paper was the same and the development, ascending, took 15 hours. Prior to spotting the paper with sample, the paper was soaked with the aqueous phase of the above mixture. Development proceeded while the paper was still wet. The chromatogram was sectioned and counted as before.

The pellet of the 40,000xg centrifugation was washed once in 5ml of buffer A then solubilized as best possible in a small volume of 0.1M

Tris-chloride pH8.3, 1% Triton X-100 and 10% sucrose. Insoluble matter was homogenized through the use of a glass rod. Aliquots of this and the supernatant were spotted onto nitrocellulose filters (Sartorius), dried at 80C and counted.

For control purposes sensitive cells were treated in the same manner. ^{3}H-AMP-DHS, prepared as described, was included also.

17. Detection of effluxing drug from cells actively accumulating drug.

Cultures were prepared as previously described for a routine uptake experiment. After twelve minutes incubation in the presence of 2.0ug/ml ^{3}H-DHS the cultures were removed from the water bath, 1.0ml extracted for the purposes of making a cell-associated label determination, then filtered through Sartorius membrane filters (47mm x 0.2u). These were washed with 20ml, and resuspended into 9.0ml, of either prewarmed NB alone or prewarmed NB with 2.0ug/ml DHS. A control was washed and resuspended into NB with 2.0ug/ml ^{3}H-DHS. Incubation was resumed at 37C in the water bath and cell-associated label determined at the specified times. Another control included similarly treated cells but exposed additionally to 1mM KCN throughout. The process of filtering and resuspending took two minutes.

18. Localization of adenylyltransferase activity in cell fractions.

R factor cells were fractionated by three different manipulations.

Osmotic shocking: Cells were shocked as previously described. Volumes of the recorded extracts were measured and assayed following overnight dialysis against 500 volumes of buffer A at 4C. Shocked cells were resuspended in small volumes of buffer A and sonicated. The suspension was centrifuged for 30 minutes at 800xg at 4C and the pellet was discarded. The supernatant was centrifuged at 40,000xg for 30 minutes and the resulting supernatant was the shocked cell fraction. The

pellet, existing now of membrane vesicles as examined by phase contrast microscopy, was solubilized on ice for fifteen minutes with the Tris-sucrose-Triton X-100 buffer described earlier. This represented a crude membrane fraction. Sonicates of a portion of the original culture were used as whole-cell controls.

Sphaeroplast formation: The recorded fractions were saved and assayed as in the preceding experiment. Sphaeroplasts were sonicated in small volumes of buffer A. The mixture was centrifuged at 40,000xg for 30 minutes, the supernatant saved and the pellet discarded. The supernatant was assumed to contain all the activity remaining associated with the sphaeroplasts upon their formation.

Isolation of membrane fractions: Purified membranes were prepared from E. coli ML strains following the methodology of Kaback (1971). Late log-phase cells, growing in minimal medium, were harvested by centrifugation, washed twice with 0.01M Tris-chloride pH8 at 4C, then resuspended at room temperature in 0.03M Tris-chloride with 20% sucrose (80ml per gram wet weight) and treated with NaEDTA pH8 and lysozyme, at final concentrations of 10mM and 0.5mg/ml respectively, for thirty minutes. Sphaeroplasts were pelleted at 16,000xg for 20 minutes and resuspended in a small volume of 0.1M potassium phosphate buffer pH6.6 with 20% sucrose and 20mM magnesium sulphate. Homogenization was attained with the aid of a motor driven teflon and glass plunger. DNase and RNase were added in concentrations such that upon the dilution of this mixture in 400 volumes of prewarmed 0.05M potassium phosphate buffer pH6.6 the concentration of each was 10ug/ml. The diluted mixture was swirled at 37C for 15 minutes then EDTA was added to 10mM. After another 15 minutes magnesium sulphate was added to 15mM. Following incubation for a further 15 minutes the membranes were col-

lected at 4C from a 30 minute spin at 16,000xg. The pellet was homogenized in 0.1M phosphate buffer containing 10mM EDTA (pH6.6) at 4C. Mg^{++}, DNase and RNase were included (20mM, 100ug/ml and 100ug/ml, respectively) and this incubated at 37C for 30 minutes with shaking. The mixture was cleared by centrifugation at 45,000xg for 30 minutes and the pellet homogenized in buffer again. Whole cells and debris were removed by repeated centrifugations (four times) at 800xg for 30 minutes. The purity of the vesicle preparations was confirmed by phase contrast microscopy. Subsequent to this the vesicles were washed 2-4 times with the phosphate and EDTA buffer. They were frozen in liquid nitrogen and stored at -70C until used. Membrane concentrations were calculated indirectly from protein concentrations assuming that the former was 1.25 times the latter (Kaback, 1971). Protein concentrations were estimated from A_{280}:A_{260} ratios.

To test for the presence of enzyme, membranes were either sonicated in buffer A or solubilized in 0.1M Tris-chloride pH8.3 with 10% sucrose and 1% Triton X-100 for 15 minutes on ice. These fractions were dialyzed overnight against buffer A (500 volumes) at 4C and assayed the next day. Membranes had been resuspended to 2.9mg/ml in each case.

19. Susceptibility of enzymes in sphaeroplasts to chemical modification by reagents unable to penetrate the cell membrane.

Sphaeroplasts were treated with two agents capable of inactivating enzymes in cell-free extracts. The agents used were SITS and trypsin.

Inactivation by SITS: Sphaeroplasts were resuspended in 9.0ml of 0.025M sodium phosphate buffer pH8 with 20% sucrose and divided into 3 2.7ml fractions A, B and C. To B was added 0.3ml of 50mM SITS in 0.025M sodium phosphate buffer pH8 to give a final concentration of 5mM. During the addition of the reagent the mixture was shaken to avoid lysis.

To A and C, equal volumes of buffer were added. Following incubation at room temperature for one hour the sphaeroplasts were collected by centrifugation and washed once with the osmotically stabilized buffer. Following resuspension in 3.0ml of phosphate buffer, the mixtures were sonicated. The supernatants from 30 minute, 40,000xg centrifugations were collected and 2.7ml of each set aside for the following treatment. To A, 0.3ml of SITS was added, to 5mM, while buffer was added in the same volumes to B and C. These were again incubated at room temperature for one hour after which the three preparations were taken and dialyzed overnight at 4C against 500 volumes of buffer A. Assays were performed the following day. C represented an untreated control, B treatment with SITS before sonication and A treatment after sonication.

Inactivation by trypsin: Fractions of sphaeroplasts were prepared and divided as above. Trypsin (2mg/ml) was added, to 20ug/ml, to B and an equal volume of distilled water was added to A and C (final volumes 3.0ml). The three were incubated at 37C for 30 minutes then rapidly cooled on ice. Trypsin inhibitor (5mg/ml) was added to B, to 50ug/ml, and equal volumes of water were added to A and C. These were allowed to sit on ice for ten minutes with periodic shaking. Sphaeroplasts were collected after this time by centrifugation at 4C and were resuspended into small volumes of 0.025M posphate buffer pH8 and sonicated. Membrane debris was sedimented and the supernatants saved. Trypsin was added, to 20ug/ml, to A and this incubated for 30 minutes at 37C. Equal volumes of water were added to B and C which were kept at 4C. After the 30 minutes inhibitor was added to A, to 50ug/ml, water added to the other two, and the three kept at 4C for ten minutes after which they were dialyzed as the fractions above. Controls included cell-free extracts treated with inhibitor alone and with enzyme-inhibitor complex

which was formed by the mixture of the two reagents, on ice, ten minutes before being added to the extracts.

20. Intracellular localization of accumulated ^{3}H-dihydrostreptomycin in R+ bacteria.

Two methods were applied in attempts to trace the intracellular fate of accumulated label. The methods, a crude fractionation and autoradiography, are described below.

Cell fractionation: ML strains were grown in 200ml of NB to 0.3 OD_{600} units/ml at which time ^{3}H-DHS (100cpm/ng) was added to 0.5ug/ml. Incubation, with shaking, continued at 37C for 30 minutes. Cell-associated label was determined then the whole culture was harvested by centrifugation and washed with standard buffer. Cells were resuspended in 0.1M Tris-chloride pH8 and 20% sucrose and sphaeroplasts were formed using the technique described earlier. Cells became spherical after 30 minutes. The suspension was frozen in liquid nitrogen then gradually thawed under warm tap water. Magnesium sulphate and DNase were added to 20mM and 100ug/ml, respectively. After incubation of the mixture for 30 minutes at 37C this was centrifuged 3 times at 800xg for 30 minutes at 4C, discarding the pellets after each run. The final supernatant was collected and small aliquots of it spotted onto nitrocellulose filters, dried at 80C and counted. The remainder was centrfuged at 40,000xg for 30 minutes at 4C, a fraction of the supernatant counted as before, and the pellet was suspended in 1ml of 5mM Tris-chloride pH8 with 20% sucrose. The resuspended pellet was rapidly diluted into 50ml of buffer A. Following a 30 minute, 40,000xg centrifugation the membrane pellet was washed once with buffer A, resuspended into a small volume of the same, and counted as before.

Autoradiography: Log-phase cells (OD_{600} units/ml of 0.3) growing

in 10ml of NB at 37C were treated with ^{3}H-DHS to 1.0ug/ml (^{3}H-DHS (3Ci/mmol) : distilled water ratio of 1:3). After 30 minutes incubation the cultures were chilled on ice, pelleted at 4C and washed twice with cold 0.05M phosphate buffer pH8, 0.5M NaCl. The washed pellets were fixed by a double fixation method: 3% glutaraldehyde (in 0.1M phosphate buffer pH7.2-7.4) at 4C overnight then 1% osmium tetraoxide in the same buffer for one hour. Samples were dehydrated by a sequential acetone treatment: 25% acetone (15 minutes), 50% acetone (15 minutes), 75% acetone (15 minutes), 90% acetone (30 minutes), 100% acetone (30 minutes) and finally with propylene oxide for 30 minutes. Preparations were subsequently left overnight in a one-to-one ratio of propylene oxide and Epon (Epon 812, Fischer Scientific) then cured the following day and night in pure Epon at 60C. Epon was prepared as per Luft (1961) combining mixtures A and B in a ratio of 3:7.

Sections were cut and placed on 1% collodin on water with a wire loop upon which the grids were then mounted. These were picked up with a glass slide and coated with L4 Ilford emulsion diluted 1:6 in distilled water. The slides were set aside at 4C for a period of 15-18 weeks. Development was with Kodak developer D-19 at 18C for 5 minutes. Samples were rinsed with distilled water, fixed for 1 minute in 25% sodium thiosulphate and washed for 5 minutes with distilled water. Finally, sections were stained, first for 10 minutes in a solution of 5% uranyl acetate in 100% methanol, washed twice with distilled water, then secondly with a solution of lead acetate (0.25g lead acetate in 50ml of preboiled distilled water followed by the addition of 5 pellets of NaOH - allowed to stand overnight before use) for 4 minutes. Stained samples were washed twice with distilled water.

Photographs of non-overlapping sections were prepared and used for

measurement purposes at a final magnification of 12,936X. The distribution of grains was determined relative to the distance from the centre of the grain, measured to the closest millimetre, to the nearest segment of the cell envelope.

RESULTS

1. Mating products.

R factor R71-a was successfully transferred from the donor, E. coli K12 J5, to four laboratory E. coli strains. The donor exhibited, as determined from Kirby-Bauer sensitivity patterns, clinical resistance to ampicillin, chloramphenicol, tetracycline and sulphonamides. It was intermediate in its resistance to streptomycin. E. coli K12 SA1306 (Rif^r Nal^r) was sensitive to the five antibiotics but was converted to clinical resistance upon the receipt of R71-a following a liquid mating with the donor strain. Similarly, the aquisition of R71-a by the three E. coli strains ML308.225 (Rif^r), K12 strain 7 (Rif^r) and K12 NR-70 (Rif^r) led to the development of resistance to each of the five antibiotics where each strain had previously been clinically sensitive. Transfer to the latter three strains was accomplished using a filter mating technique. Liquid matings between the donor and the ML recipient had earlier proven unsuccessful whether the incubation period was 90 minutes or 18 hours. E. coli K12 NR-70, as opposed to its parent, displayed slight growth on TSA with 12.5ug/ml DHS. Selection of transconjugants (recipients of the R factor) for both these strains was, accordingly, performed on TSA with 50ug/ml DHS upon which neither strain would grow.

2. Minimum inhibitory concentrations.

Dihydrostreptomycin MICs were determined in those broths that would be employed throughout the study. Values are presented in table I. Strains that possessed the R factor were intermediate in resistance when compared to the strA mutants of the same strain (Str^r strains as indicated in the tables), a relationship which was apparent in both broths

though the degrees of resistance varied from one to the other, being greater in TSB. Protection of the ML strain from the effects of DHS by chromosomal mutation or R factor incorporation was noticeably less than that with the K12 strain.

3. Adenylyltransferase activity in cell-free extracts.

Extracts of R+ bacteria were prepared through osmotic shocking and sonication and these were assessed for enzyme activity. As indicated in table I the R factor donor had the capacity to inactivate DHS by adenylylation. Recipient strains aquired this capacity with the transfer of R71-a. Positive activity denotes a positive difference between levels of binding of ^{14}C-ATP to phosphocellulose paper in the test and control experiments. When activity was evident in cell-free extracts of sonicated cells it was similarly noted in the osmotic shockates of the same strain and vice versa.

4. Binding of DHS to crude ribosomal preparations.

Qualitative determinations were made to assess the capacity of ribosomes from R+ strains to bind DHS. It was conceivable that during the selection of these strains ribosomal mutants were isolated which carried the R factor. The results (table II) show that this was not the case. Ribosomes from these strains are at least as competent of binding DHS as those from R- organisms. Chromosomally resistant bacteria (Str^r strains) characteristically possess ribosomes devoid of this capacity. Free-DHS measurements were made from those chambers opposite the ribosomes; free-plus bound-DHS from the ribosome-containing chambers and the difference represented bound DHS. Dialysis was continued for up to 48 hours.

Calculations based on the concentrations as they were presented in the methods indicate that the binding of 50ng of DHS under the specified conditions would represent a final ratio of one ribosome per 0.55 molecules

TABLE I. Dihydrostreptomycin MICs for E. coli strains in nutrient and trypticase soy broths and the capacity of cell-free extracts to inactivate DHS by adenylylation.

Strain	MIC ug/ml		Adenylyltransferase activity
	NB	TSB	
K12 J5 R71-a	1.0	ND	+
K12 SA1306 Str^s	0.25	10	-
K12 SA1306 R71-a	15.6	1250	+
K12 SA1306 Str^r	250	>20000	ND
ML308.225 Str^s	0.5	10	-
ML308.225 R71-a	2.0	20	+
ML308.225 Str^r	62.5	ND	ND

ND : not determined, + : positive, - : negative

TABLE II. *In vitro* binding of DHS to crude ribosomal preparations from *E. coli* strains.

Strain	ng DHS		
	Free-DHS	Free- plus bound-DHS	Bound-DHS
K12 SA1306 Str^s	418	470	52
K12 SA1306 Str^r	404	403	0
K12 SA1306 R71-a	581	640	59
ML308.225 Str^s	491	537	46
ML308.225 Str^r	491	473	0
ML308.225 R71-a	487	563	76

Free-DHS : total drug in dialysis cell opposite ribosomes; Free- plus bound-DHS : drug in dialysis cell with ribosomes; Bound-DHS : positive difference between the two.

of dihydrostreptomycin. Chang and Flaks (1972) achieved a ratio of 0.82 molecules per ribosome.

5. Mutagenesis of E. coli K12 SA1306 R71-a.

Selection was for mutants that manifested reduced resistance towards DHS which, in the final analysis, would be reflected in a modification of the inactivating enzyme. EMS mutagenesis, as described, resulted in a survival rate of just under 1%.

Several thousand colonies were patched and from these none failed to grow on TSA supplemented with 12.5ug/ml DHS. However, four colonies did show reduced growth and these were selected and isolated. Sensitivity profiles indicated that these strains had retained the R factor and the levels of resistance to all antibiotics except for streptomycin. Zones of inhibition, in the latter case, were enlarged though the organism still retained intermediate resistance to the drug. Tube MICs confirmed the enhanced susceptibilities to DHS characteristic of these isolates. One, variant SR15, was selected for future examination.

Table III notes what effect the mutations had on the MICs for DHS. The increase in sensitivity shown has been mirrored, with marked similar proportions, in a reduced MIC for spectinomycin. MICs do, however, remain higher than those of the R- strains. Osmotic shockates of the mutant repeatedly demonstrate a reduced specific activity of adenylyltransferase amounting to just below half that of the wild-type. It is not known whether the reduced specific activity was a function of altered binding specificities or a reduction in the amount of the enzyme synthesized. Revertants were obtainable that possessed DHS MICs identical to that of the R+ wild-type at a frequency of one in 10^5 organisms.

6. R factor stability.

Throughout the course of the investigation, there were no overt signs

TABLE III. Adenylyltransferase activity of EMS-mutagenesis isolate *E. coli* K12SA1306 R71-a var. SR15 relative to the fully-resistant R^+ parent and the sensitive R^- strain.

Strain	MICs ug/ml		Sp. act. EU/mg prot. adenylyltransferase
	DHS	Spectinomycin	
K12SA1306 Str^S	0.25	4.0	0
K12SA1306 R71-a	15.6	250	6.4×10^{-2}
K12SA1306 R71-a var. SR15	2.0	32	3.1×10^{-2}

TABLE IV. Percentage of E. coli cells retaining the R factor after 10 to 15 generations of growth in DHS-free (final) and overnight growth in DHS-containing (initial) media.

Strain	Initial population	Final population
K12 SA1306 R71-a	100	100
K12 SA1306 R71-a var. SR15	100	100
ML308.225 R71-a	100	100

that the R factors were anything but stably incorporated into the bacteria. To confirm that this was indeed so, a test for R factor stability was applied. The results, as presented in the fourth table, substantiate this observation. No indications existed suggesting the instability of the R factors.

7. Dihydrostreptomycin uptakes in whole cells.

Uptakes were performed on log-phase cultures growing in NB. It is clearly shown in the fourth figure that the accumulation of DHS be E. coli K12 SA1306 (Rif^r Nal^r) at 1.0ug/ml DHS occurs in three stages. The first was a rapid binding, essentially occurring within the time required to make the initial reading, some 40 seconds. A lag phase, or at most a very low rate of accumulation, followed for the next five to seven minutes. The third phase was characterized by a greatly enhanced, linear rate of accumulation. After 30 minutes incubation the organisms had accumulated within the cell 430ng DHS per ul of cell water assuming 2.7ul cell water per mg dry weight of cells. The dry weight of E. coli K12 SA1306 was found to be 0.18mg/OD_{600}/ml. The extracellular concentration was 1.0ng/ul. Deviations in the logarithmic growth of the organism, signaling growth inhibition, were first noted at the tenth minute.

E. coli K12 SA1306 R71-a, at the same DHS concentration, one which is intermediate to the MICs of the two strains, fails to show the third phase of uptake. At the same time there was no deviation, over-and-above that of the controls, from logarithmic growth. Accumulation of the drug was evident over the thirty minute period, as an extension of the second phase of uptake. This is more clearly seen in the next figure. Nonetheless, after the thirty minutes, R- cells have taken up more than ten times the amount of drug that the R+ cells have.

At 2.0ug/ml (figure 5), the significant differences in the accumulation

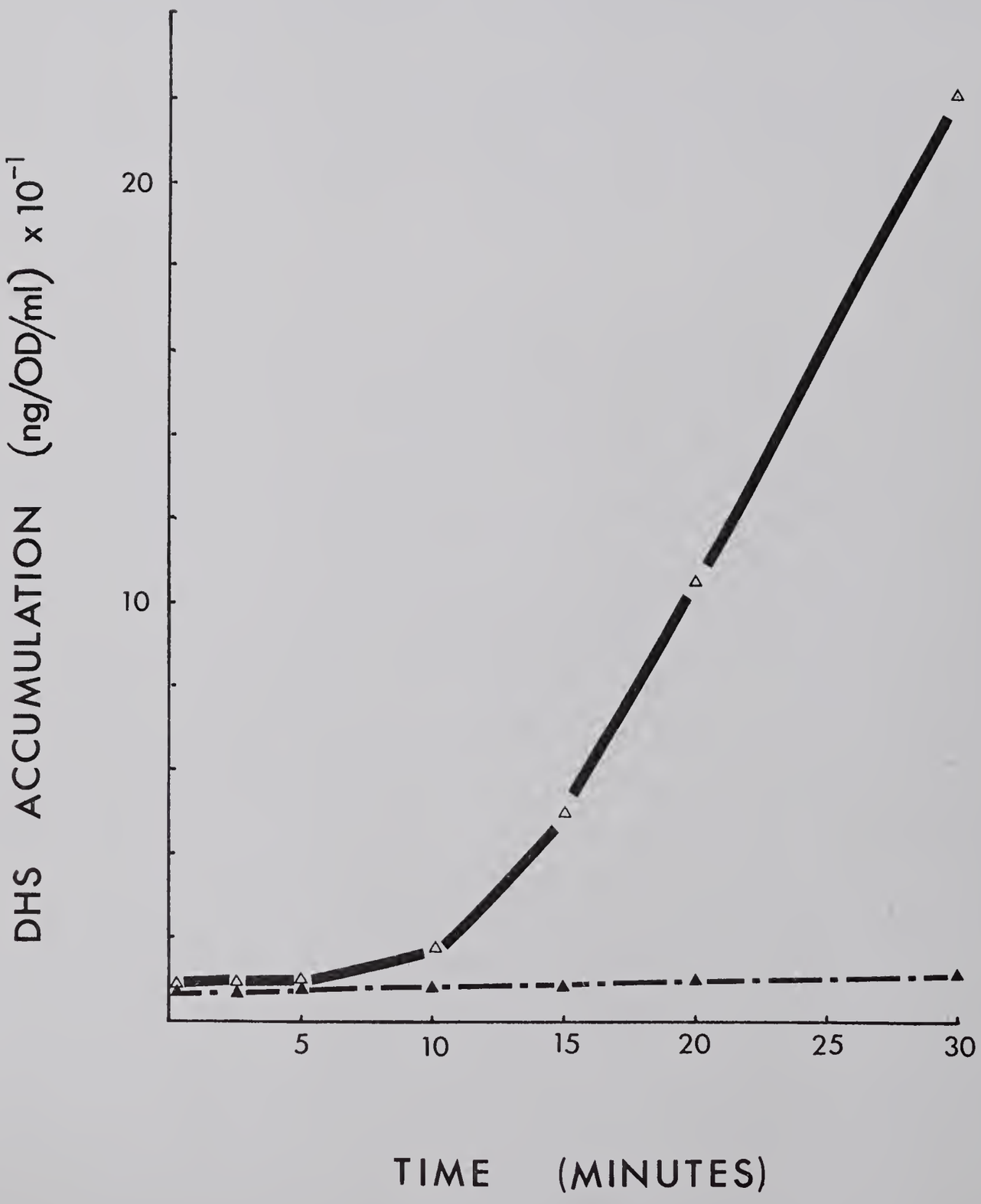

DHS ACCUMULATION (ng/OD/ml) x 10⁻¹
20
10
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 4: Accumulation kinetics of DHS at 37C at 1.0ug/ml DHS in NB by E. coli strains K12 SA1306 Str^S (△▬▬△) and K12 SA1306 R71-a (▲▬ ▪ ▬▲).

of DHS by R- bacteria are a shortening of the second phase and an acceleration of the third phase rate. R+ cells begin to show signs of having entered a third phase of uptake, starting at 15 minutes, but the rate was much reduced in comparison to that of the R- strain. No deviation in growth rate was observed. The second phase rate in these organisms also appears greater. In the same graph is represented the accumulation profile of the sensitive cell growing in the presence of 1mM KCN. Third phase kinetics was obviously lacking and, when compared to the R+ strain, so was the second phase. R+ cells treated with KCN take up drug in the same manner. Only the initial binding was evident in these poisoned cells. Finally, it can be seen that the accumulation of drug by the mutant SR15 was intermediate to the R- and R+ populations with respect to total uptake, the rate and the onset of the third phase of uptake.

As the concentration of DHS approaches the MIC of the R+ strain (figure 6), the uptake profile of this strain changes predictably. The third phase was initiated earlier and progressed at a greater rate. The second phase, now shorter in duration, was apparently at an enhanced rate. R- cells begin to show signs of saturation at these concentrations as transport was no longer linear after 15-20 minutes.

Similar descriptions can be afforded the uptake profiles for E. coli ML308.225 with and without the R factor. Once again, at drug concentrations intermediate to the MICs of the two strains (figure 7) R- cells accumulate more drug, 150ng/ul cell water versus 35ng/ul for the R+ cells. There was no evidence of uptake in the third phase by R+ cells and no signs of growth inhibition.

8. Dihydrostreptomycin uptakes in sphaeroplasts.

An ML strain of E. coli was used for the preparation of sphaeroplasts in light of the documented ease with which the conversion can be made.

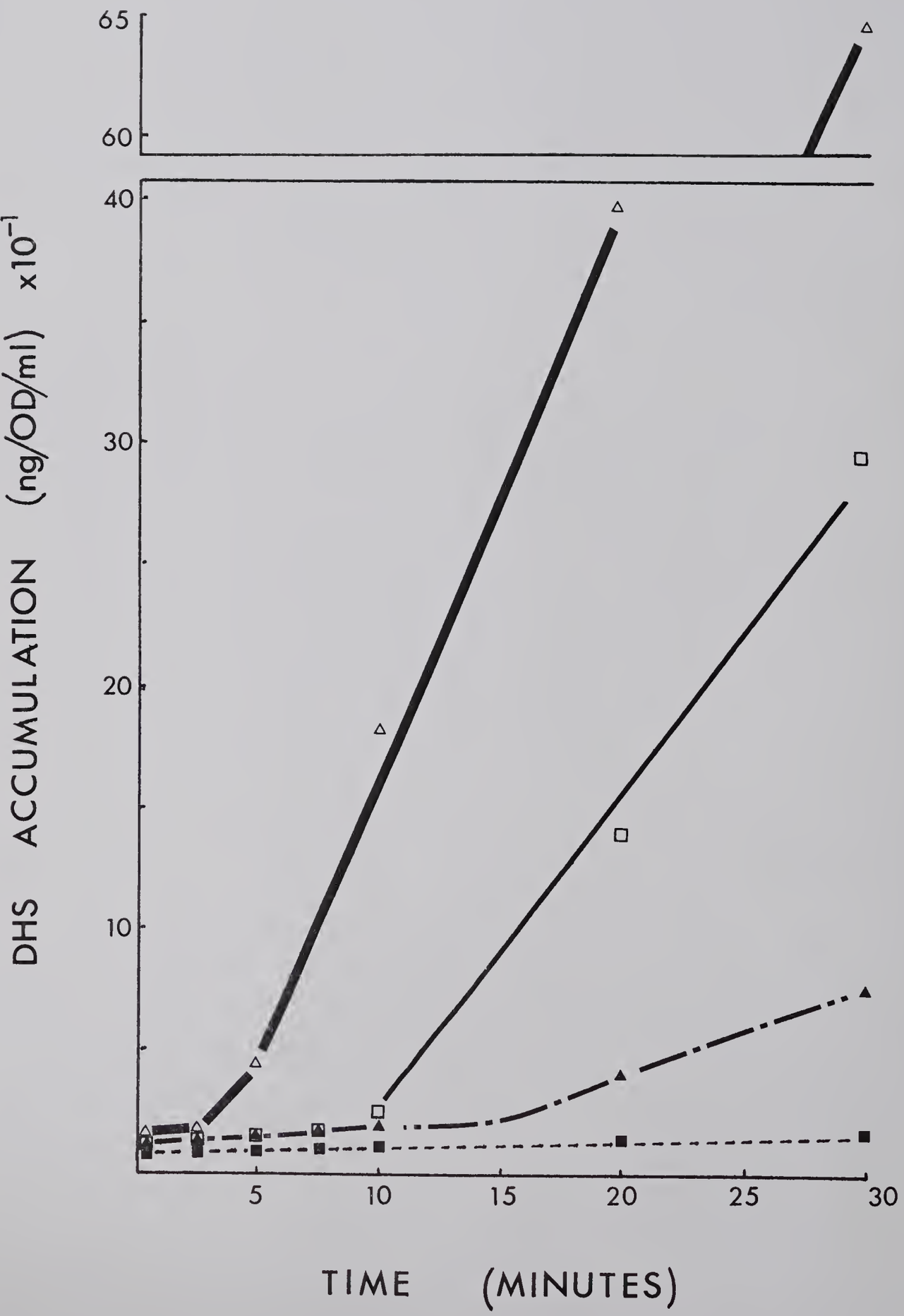

65
60
40
30
20
10
DHS ACCUMULATION (ng/OD/ml) x10⁻¹
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 5: Accumulation kinetics of DHS at 37C at 2.0ug/ml DHS in NB by E. coli strains K12 SA1306 Str^S (△▬▬△), K12 SA1306 Str^S with 1mM KCN (■·-·-·-·■), K12 SA1306 var. SR15 (□ ▬▬ □) and K12 SA1306 R71-a (▲ ▬·▬ ▲).

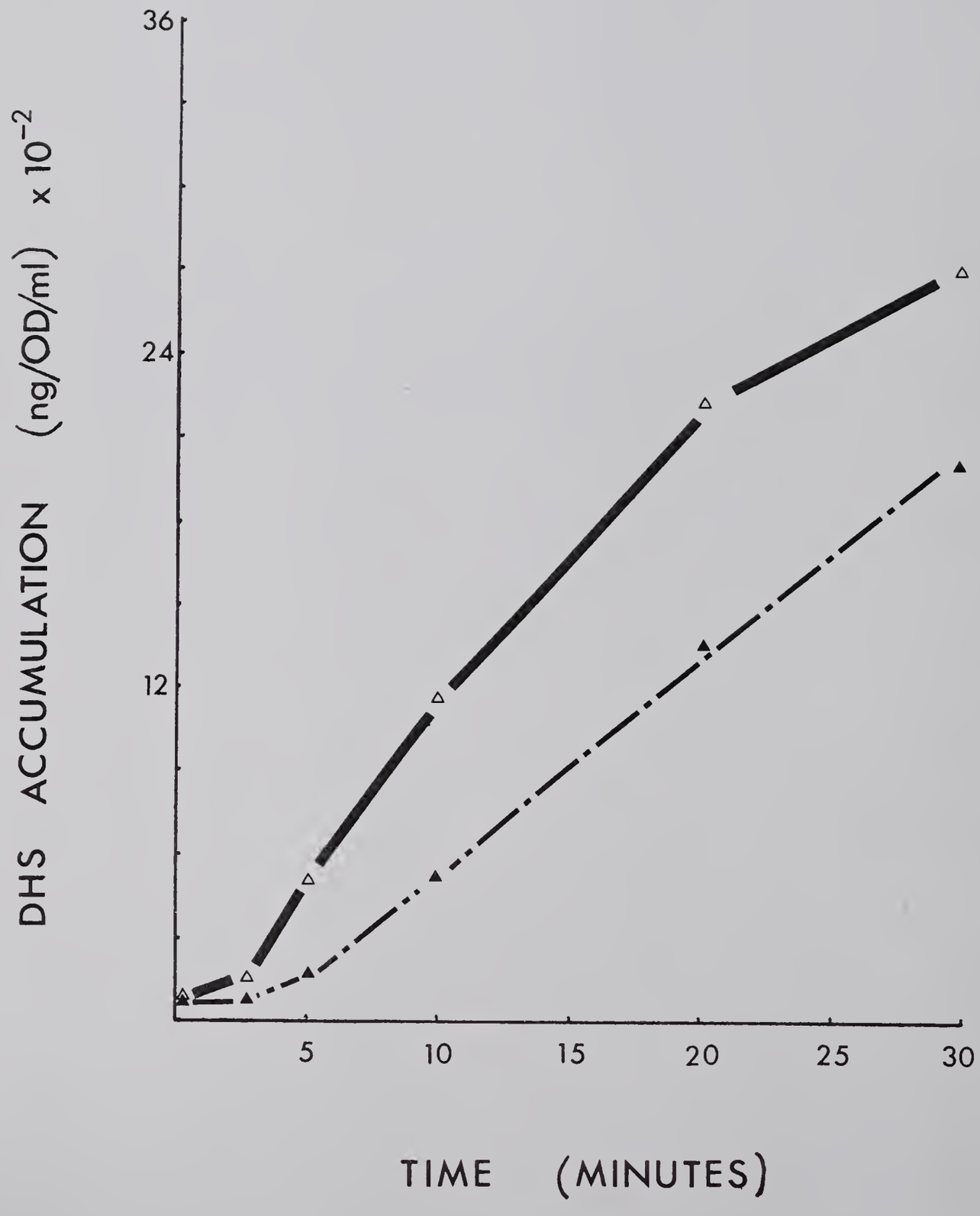

DHS ACCUMULATION (ng/OD/ml) x 10⁻²
36
24
12
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 6: Accumulation kinetics of DHS at 37C at 10ug/ml DHS in NB by E. coli strains K12 SA1306 Str^S (△▬▬△) and K12 SA1306 R71-a (▲▬·▬▲).

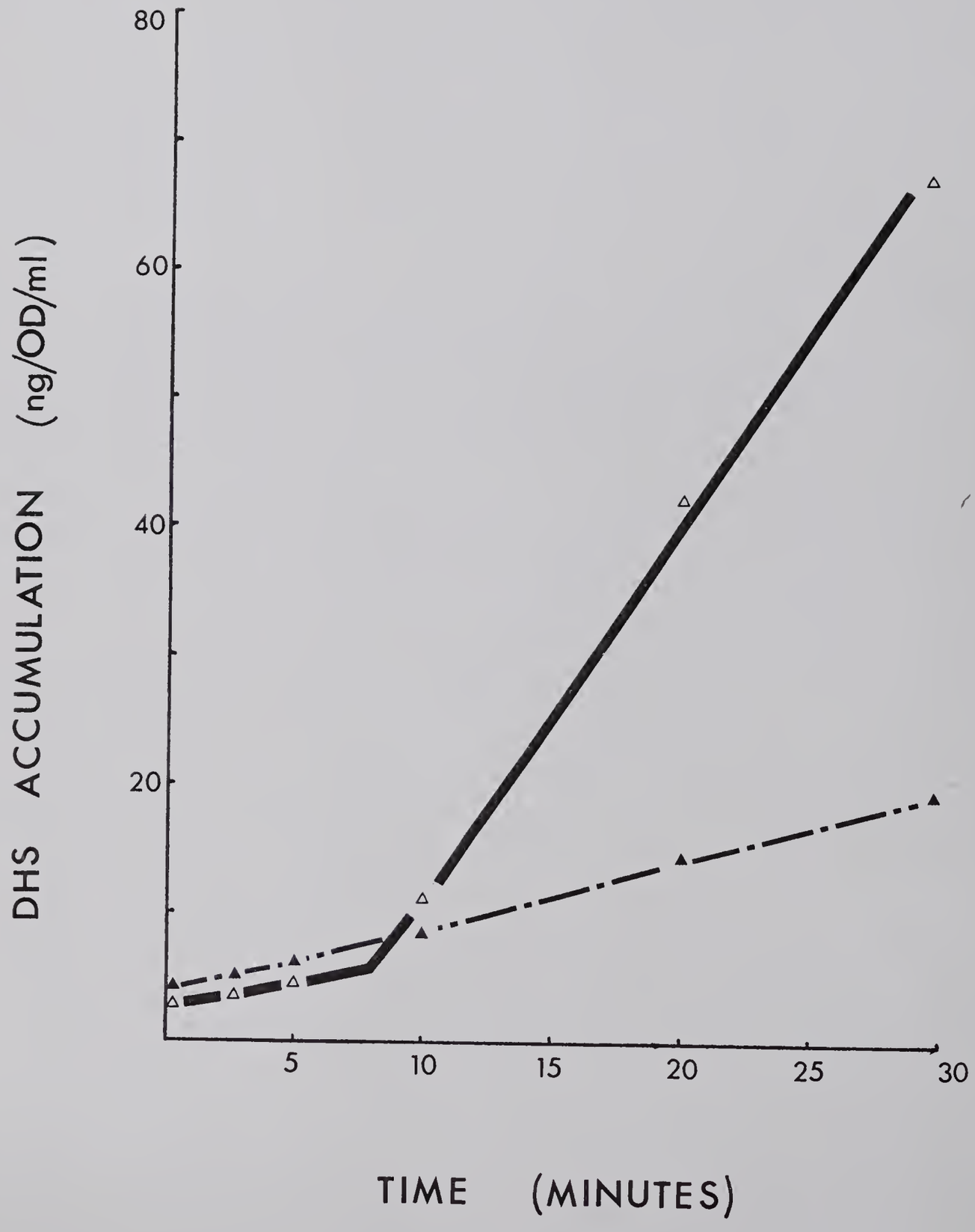

80
60
40
20
DHS ACCUMULATION (ng/OD/ml)
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 7: Accumulation kinetics of DHS at 37C at 0.5ug/ml DHS in NB by E. coli strains ML308.225 Str^S (△▬▬△) and ML308.225 R71-a (▲▬·▬▲).

(Kaback, 1971 and Weiss, 1976). Under the conditions described in the Methods, the preparation of good quality sphaeroplasts was a reliable process. They were durable in that extensive lysis could be avoided with gentle handling. Preparations were essentially void of rod-shaped forms (<1%) after 20 to 30 minutes incubation in the presence of EDTA and lysozyme. Electron microscopic examination verified the absence of gross cell wall structures (polar caps, outer membrane) remaining attached to these spherical forms.

Accumulations of DHS in sphaeroplasts (figure 8) were qualitatively similar to those in whole cells. The third phase of uptake seen with R-, and not R+ sphaeroplasts, began at the same time as it did in whole cells. Total uptake by each strain differs substantially and the differences can be directly traced to the absence of third phase kinetics in the R+ strain. This strain does accumulate drug linearly throughout the 30 minutes by second phase kinetics analogous to whole cells.

The OD_{600} of the R+ sphaeroplast suspension remained constant throughout the experiment. In contrast, drops in OD_{600} compared to untreated controls, of 10 to 30% were observed with suspensions of R- sphaeroplasts. This is most clearly shown if the sphaeroplasts are first allowed to equilibrate in the prewarmed broth for 15-20 minutes in order to establish a constant OD_{600}. DHS was added in small volumes so as to upset the osmotic balance as little as possible.

9. Detection of modified drug in the culture medium.

Medium filtrates of four hour cultures of R+ bacteria growing in TSB with 50ug/ml of ^{3}H-DHS were assayed by paper chromatography for the presence of adenylylated dihydrostreptomycin. The chromatography system applied allowed for the greatest separation of modified and unmodified drug of those systems tested. The Rf for AMP-DHS was very small and the

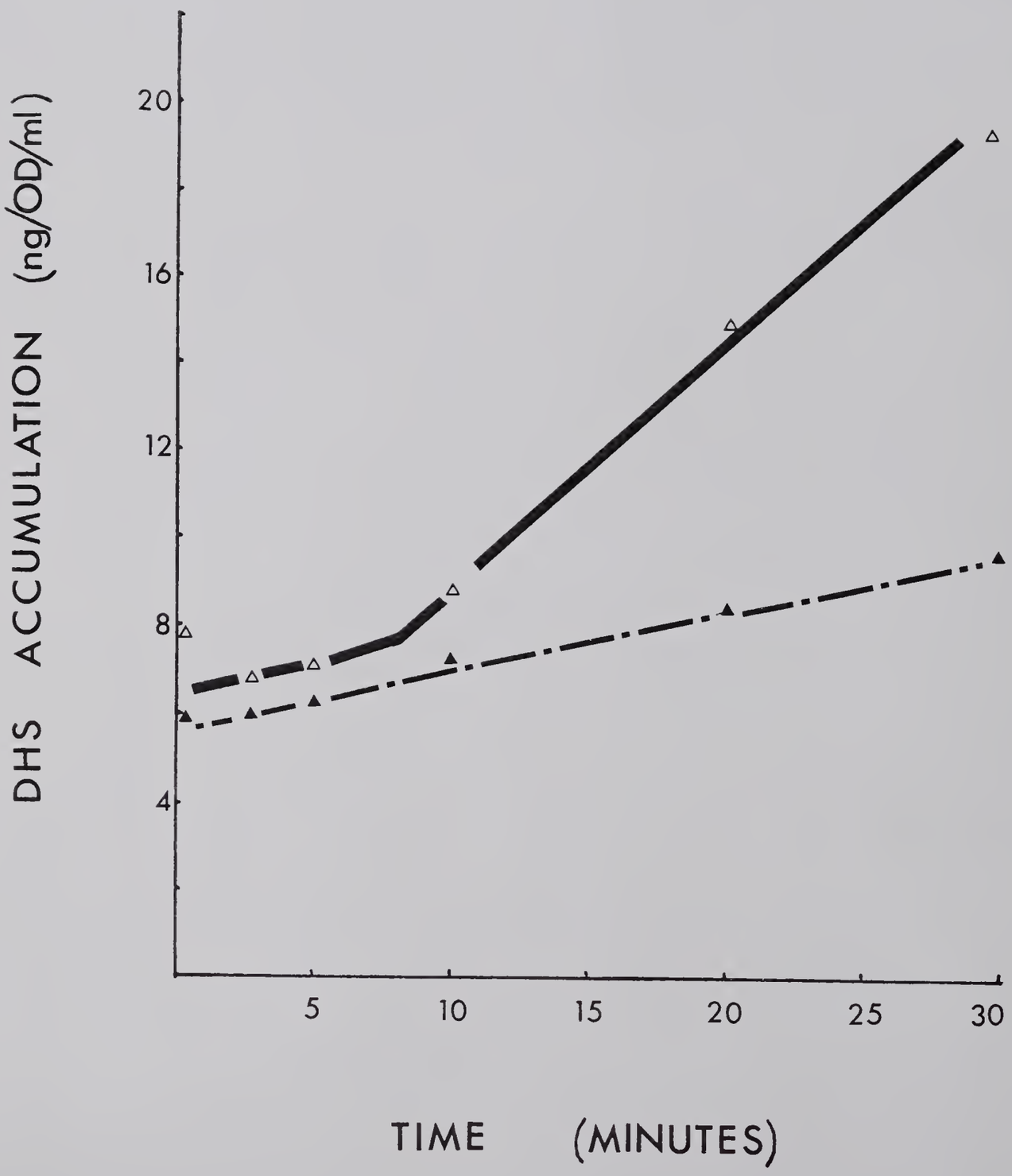

DHS ACCUMULATION (ng/OD/ml)
20
16
12
8
4
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 8: Accumulation kinetics of DHS at 37C at 0.5ug/ml DHS in NB with 20% sucrose by sphaeroplasts of E. coli strains ML308.225 Str^S (△▬▬△) and ML308.225 R71-a (▲▬·▬▲).

development time long (48 hours).

The histograms of figure 9 represent the chromatographic profiles of the filtrates. Cultures of the Str^r strain were used as controls. No evidence of modification, in the region where AMP-DHS would migrate existed. The system would have been expected to have reliably detected an amount of AMP-DHS as low as 1% of the total DHS present. The overnight incubation of similarly treated cultures yielded comparable results. The secondary peak migrating to the thirteenth fraction was present, in most occasions, at levels varying from 5% to 20% of the total label. ^{3}H-DHS, alone and in TSB, resulted in similar profiles though only a single peak was observed in a second paper chromatography system to be discussed later. The identity of the material constituting this second peak was not investigated. Double-peaking of DHS has been observed when in the presence of certain salts (Peterson and Reineke, 1950).

10. Detection of effuxing drug from cells actively accumulating DHS.

E. coli K12 SA1306 R71-a was incubated for 12 minutes in the presence of 2.0ug/ml ^{3}H-DHS, filtered, washed and then resuspended in NB with DHS of the same concentration. An initial rapid loss of label from the cells was followed by a plateau period during which there was very little label lost. The accumulation of drug can be shown to continue throughout the experiment after a brief lag as shown partially by the top curve of figure 10. Cells treated with KCN through every stage of the experiment demonstrated the initial rapid loss, but also lost drug continually after this though at a much reduced rate. The amount of drug accumulated by these cells was typically less than that taken up by unpoisoned cells. Similar results were found when the cells were resuspended into NB without any added DHS.

The manipulative process of filtering, washing and resuspending took

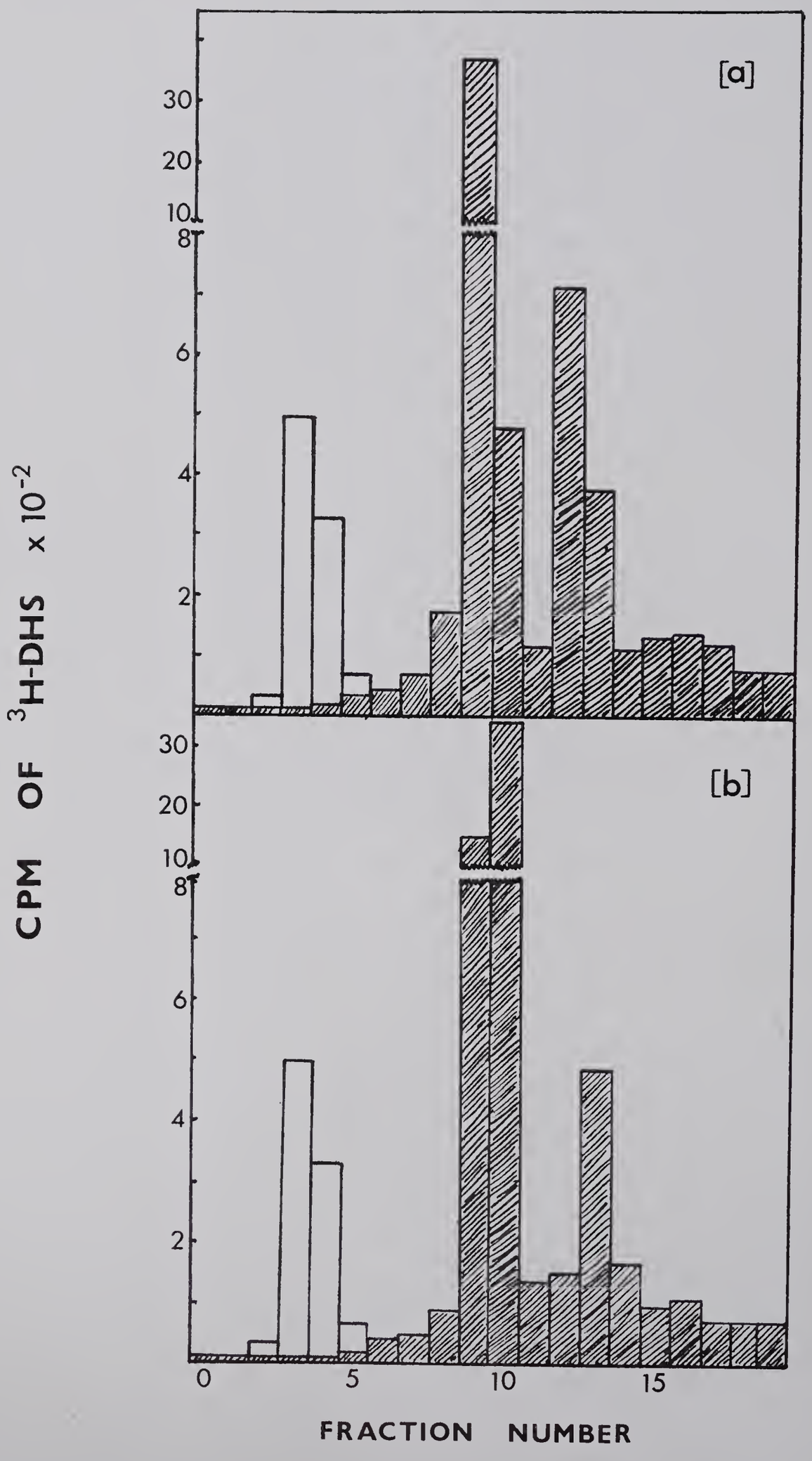

[a]
[b]
30
20
10
8
6
4
2
0
5
10
15
CPM OF ^{3}H-DHS x 10^{-2}
FRACTION NUMBER

FIGURE 9: Chromatographic profiles of culture filtrates of E. coli strains K12 SA1306 R71-a (a) and K12 SA1306 Str^r (b) grown for 4 hours at 37C in TSB with 50ug/ml ^{3}H-DHS (hatched bars). The migration of ^{3}H-AMP-DHS is shown, to an arbitrary scale, by the unhatched bars. Fractions are one centimetre strips. O represents the origin.

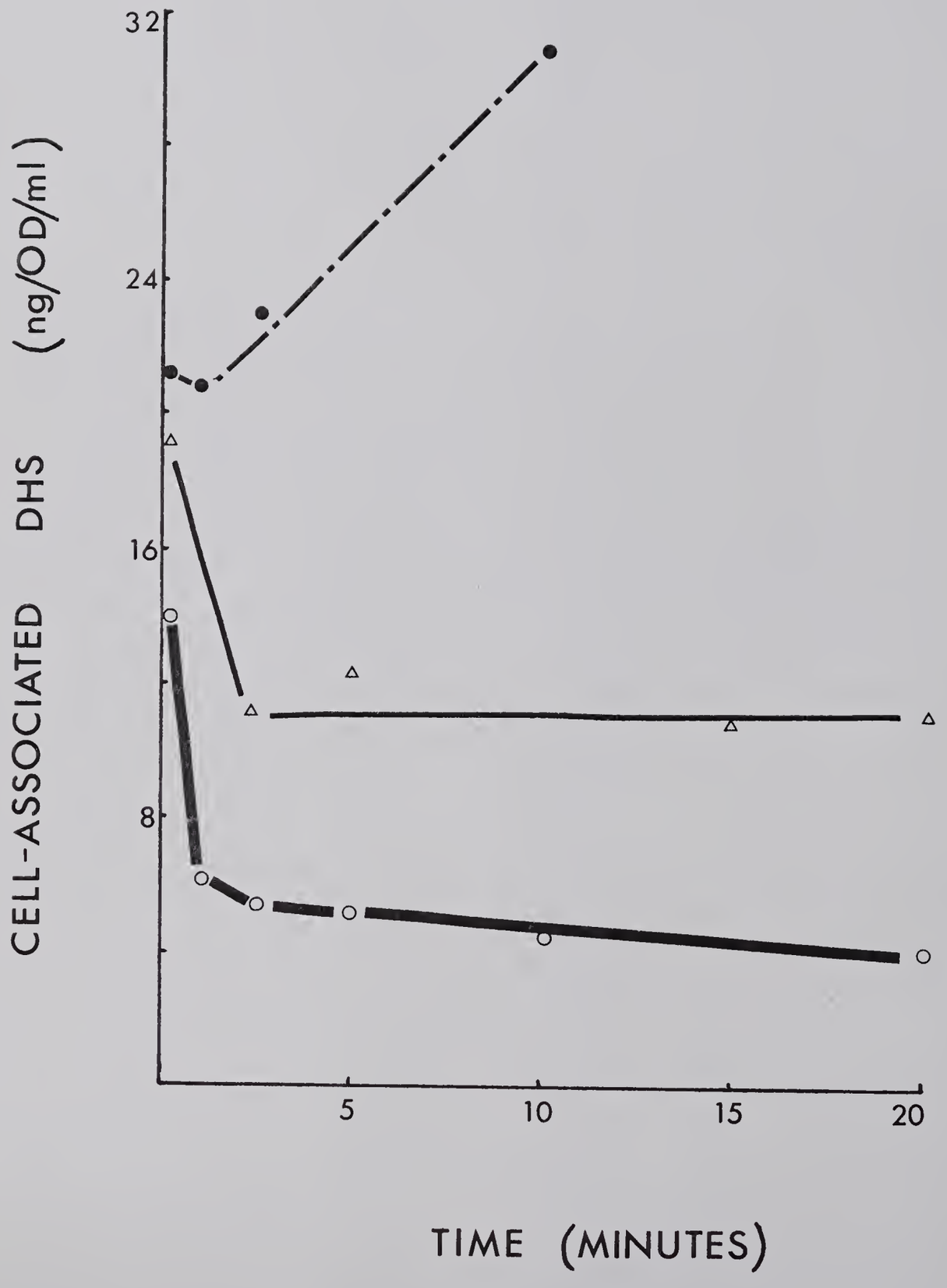

32
24
16
8
CELL-ASSOCIATED DHS (ng/OD/ml)
5
10
15
20
TIME (MINUTES)

FIGURE 10: The release of cell-associated label at 37C from E. coli K12 SA1306 R71-a, preloaded with ^{3}H-DHS from 12 minutes growth in NB with 2.0ug/ml ^{3}H-DHS, after resuspension into NB with 2.0ug/ml unlabelled-DHS (△——△). Controls include cells resuspended back into 2.0ug/ml ^{3}H-DHS (●—·—●) and cells treated with 1mM KCN throughout (○——○).

two minutes. Inevitably, variable amounts of drug were lost during the process. Zero readings in figure 10, as a result, represent the higher of the two values of cell-associated label determined before and after transfer.

11. Detection of intracellular inactivated drug.

Aliquots of cell-free sonicates of R+ bacteria, grown in the presence of 1.0ug/ml ^{3}H-DHS, were chromatographed in an effort to identify the label accumulated by these strains during the second phase of uptake. The chromatographic system in this instance allowed for a shorter development time, the Rf of modified drug was greater and though not as well separated the two forms of drug were easily identifiable. Figures 11 and 12 represent the paper chromatogram profiles of the results as histograms. As a control similarily treated R- organisms were used.

After 15 minutes incubation with ^{3}H-DHS, adenylylated-DHS was evident in the cells. It represented approximately 25% of the total amount of label accumulated (calculated from the area of the bars). After thirty minutes the relative amount of AMP-DHS rose to almost 40%. No peak analogous to AMP-DHS was visible with the extracts from the R- cells. Only one peak, that corresponding to DHS, was observed. 100ul and 20ul of the extracts from R+ and R- cells, respectively, were chromatographed.

Most of the label associated with the cells after the 30 minute period has been released into the sonicate supernatant (table V). At thirty minutes, 93% of the total label from R+ cells has been represented in the chromatograms of the previous two figures. A relatively constant amount remained associated with the particulate matter. The difference in the total uptakes of the two organisms is clear from this table.

12. Localization of cell-associated drug.

Two methods were applied to help ascertain the intracellular fate of

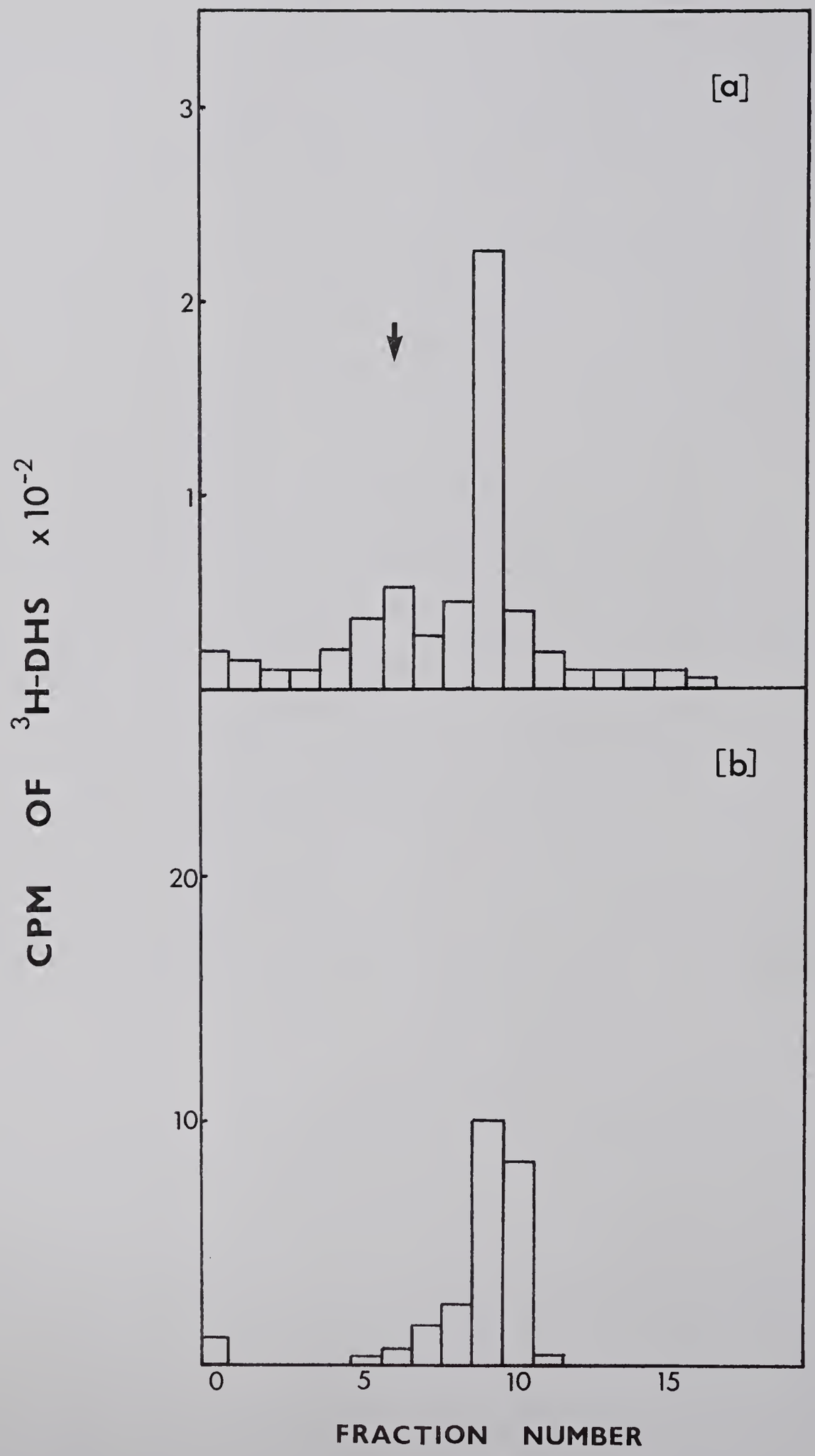

[a]
[b]
3
2
1
20
10
0
5
10
15
CPM OF ^{3}H-DHS x 10^{-2}
FRACTION NUMBER

FIGURE 11: Chromatographic profiles of cell-free sonicates of *E. coli* strains K12 SA1306 R71-a (a) (100ul spotted) and K12 SA1306 Str^s (b) (20ul spotted) grown for 15 minutes at 37C in NB with 1.0ug/ml ^{3}H-DHS. Fractions are one centimetre strips. O represents the origin. The arrow indicates the peak migration of ^{3}H-AMP-DHS when chromatographed alone.

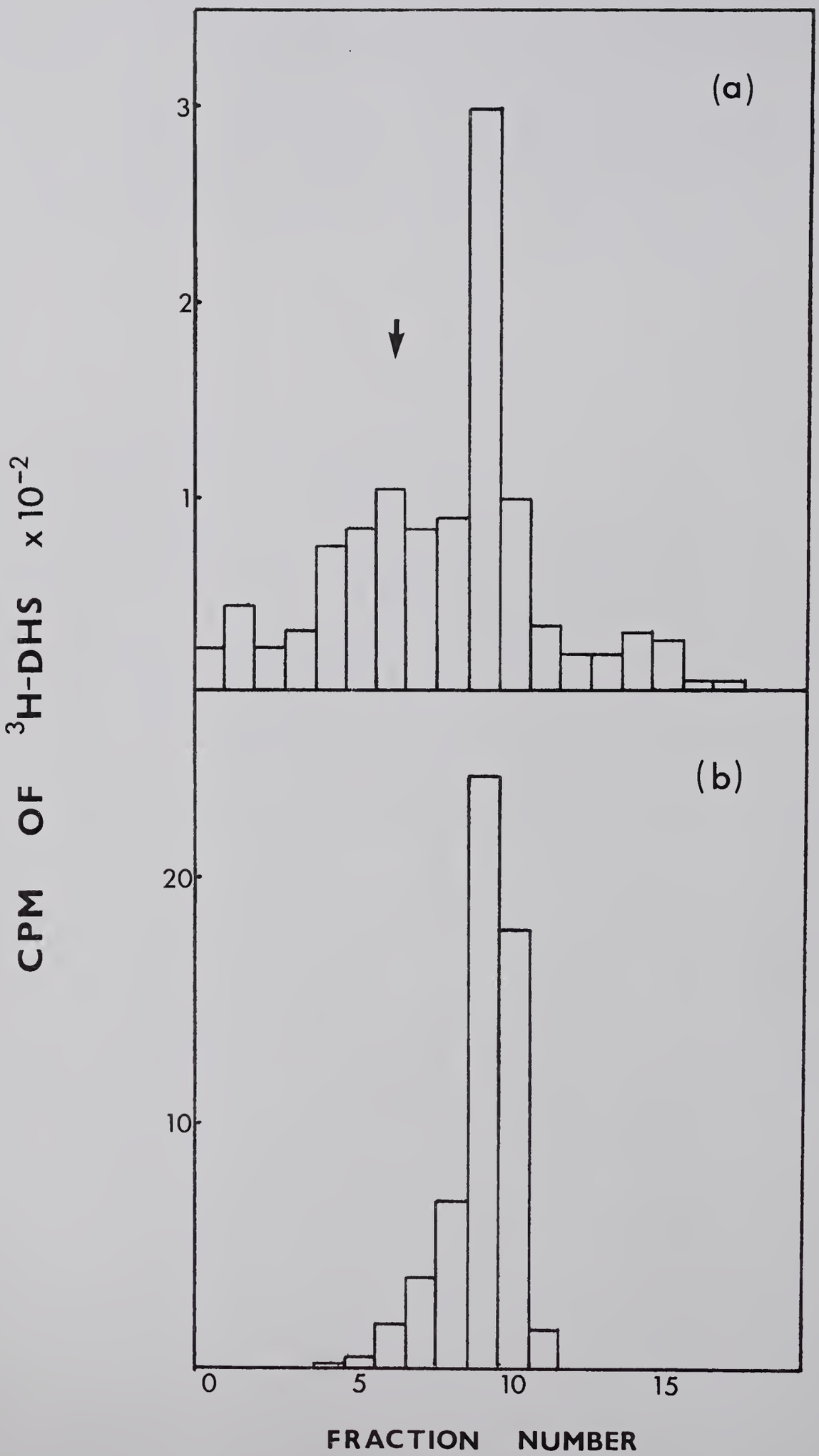

(a)
(b)
3
2
1
20
10
0
5
10
15
CPM OF ^{3}H-DHS x 10^{-2}
FRACTION NUMBER

FIGURE 12: Chromatographic profiles of cell-free sonicates of E. coli strains K12 SA1306 R71-a (a) (100ul spotted) and K12 SA1306 Str^S (b) (20ul spotted) grown for 30 minutes at 37C in NB with 1.0ug/ml ^{3}H-DHS. Fractions are one centimetre strips. O represents the origin. The arrow indicates the peak migration of ^{3}H-AMP-DHS when chromatographed alone.

Table V. Release by sonication of accumulated label from E. coli K12 SA1306 strains after periods of growth in NB with 1.0ug/ml ^{3}H-DHS. Supernatants were chromatographed to determine the identity of the label (figures 11 and 12).

		DHS (ng/OD_{600}/ml whole cells)	
E. coli strains	time (minutes)	40,000xg supernatants	40,000xg pellets
K12 SA1306 R71-a	15	5.6	1.0
	30	12.4	0.9
K12 SA1306 R^{-}	15	103	1.0
	30	266	2.1

accumulated label in R+ organisms that had taken up drug during the first and second phases only. The first method involved the crude fractionation of E. coli ML308.225 R71-a which had been grown in NB with ^{3}H-DHS (0.5ug/ml) for 30 minutes. After the freeze rupture of the sphaeroplasts prepared from these cells the label was essentially released into the soluble fraction (table VI). Less than 2% of the total accumulated label was traceable to the crude membrane fraction. Indeed, the same was true for R- cells. The formation of sphaeroplasts of treated cells was at least 99% complete. After the freezing and thawing there was a complete conversion to vesicles.

The second method involved the autoradiographic detection of ^{3}H label in whole cells of E. coli K12 SA1306 R71-a grown for thirty minutes in NB with 1.0ug/ml ^{3}H-DHS. Incubation under these conditions does not lead to the active accumulation of drug by third phase kinetics, only second phase kinetics (figure 4). Sensitive cells, to the contrary, in accumulating more drug do so predominantly by third phase kinetics. Figure 13 indicates that in spite of these differences, the amount of drug accumulated and the means by which its achieved, the distribution of drug in the cells is similar. The majority of the drug is wall-membrane complex-associated. Only a small fraction, suggested by the skewing of the graphs in the direction of the cells interior, appears essentially "internal". Approximately 200 grains were considered for the purposes of constructing each graph. Grains, on the average, were 3mm in diameter. The only difference between the two preparations was quantitative, more grains being associated with the sensitive organisms.

13. Localization of adenylyltransferase activity in cell fractions.

The release of adenylyltransferase was followed during several different fractionation procedures involving R+ cells.

TABLE VI. Release of accumulated label by the freeze-rupture of sphaeroplasts formed from E. coli ML308.225 grown for thirty minutes in NB with 0.5ug/ml ^{3}H-DHS.

		DHS (ng/OD_{600}/ml whole cells)		
E. coli strain	time (minutes)	800xg supernatant	40,000xg supernatant	40,000xg pellet
ML308.225 R71-a	30	28.1	27.8	0.31
ML308.225 R-	30	54.4	51.9	0.74

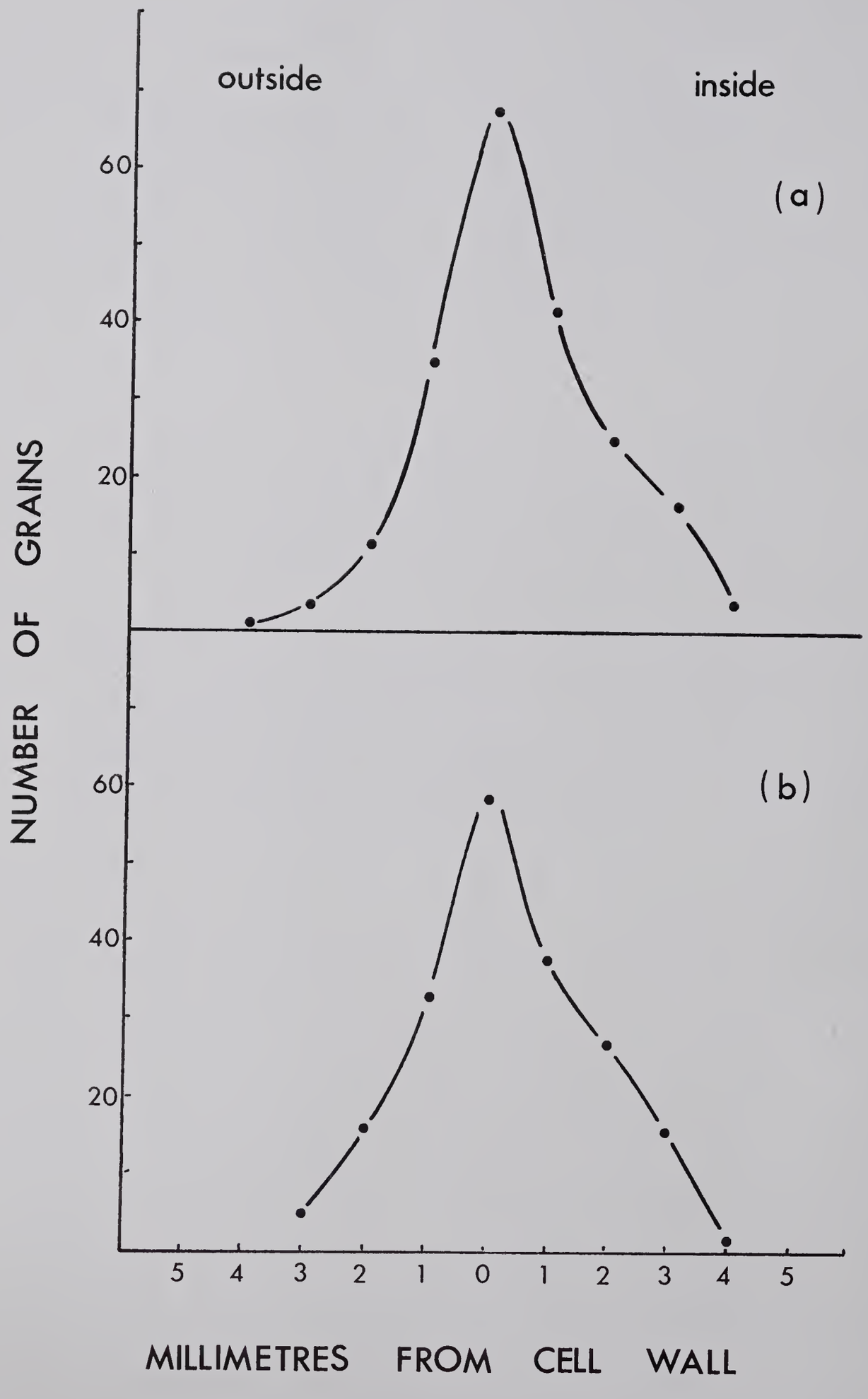

outside
inside
(a)
(b)
60
40
20
60
40
20
NUMBER OF GRAINS
5 4 3 2 1 0 1 2 3 4 5
MILLIMETRES FROM CELL WALL

FIGURE 13: Cellular localization of tritium-labelled dihydrostreptomycin in E. coli K12 SA1306 R71-a (b) and E. coli K12 SA1306 Str^s (a) having grown in NB with 1.0ug/ml ^3H-DHS at 37C for thirty minutes. Grains are grouped by distances (in millimetres) from the cell wall-membrane complex (0).

The osmotic shocking of E. coli K12 SA1306 R71-a resulted in the near quantitative release of adenylyltransferase and alkaline phosphatase. The release of internal contents, as indicated by the activity of beta-galactosidase, amounted to 40% of total (table VII). Crude membrane fractions, of shocked cells consisting primarily of membrane vesicles as observed by phase contrast microscopy, retained only contaminating levels of the three enzymes. Solubilization of membranes, as described in the Methods, would not be expected to have a deleterious effect on the activity of adenylyltransferase were it to exist in some of these extracts. Sonicated extracts had been made to 1% Triton X-100 and 10% sucrose, kept on ice for 15 minutes then dialyzed overnight against 500 volumes of buffer A, without the appreciable loss of any activity. The activities recorded were 15.3EU/ml for the untreated R+ extract and 13.6EU/ml for the treated extract.

Sphaeroplasting of E. coli ML308.225 R71-a released alkaline phosphatase quantitatively only (table VIII). Adenylyltransferase and lactate dehydrogenase were retained by sphaeroplasts, until sonication, to comparable degrees. Lactate dehydrogenase was assayed because these strains were Lac^- (z^-).

Membrane vesicles of E. coli ML308.225 R71-a were isolated and assayed for enzyme activity. Negative results were obtained in all instances when values were compared to controls (vesicles from R- cells). The membrane yield was 8.5mg per 75mg dry weight of starting material.

14. Susceptibility of adenylyltransferase to chemical modification in sphaeroplasts.

E. coli ML308.225 R71-a sphaeroplasts were treated before and after sonication with two chemical reagents known to inactivate adenylyltransferase in cell-free extracts, SITS and trypsin. Table IX records the

Table VII. The release during the osmotic shocking of E. coli K12 SA1306 R71-a of adenylyltransferase compared to marker enzymes.

Cell fraction	Enzyme activity *		
	AAD-(3")	Alkaline phosphatase	Beta-galactosidase
cell-free sonicate	22.5	30.1	90.1
sucrose-EDTA supernatant	1.4	2.9	0.0
shock fluid	20.7	24.0	35.8
shocked cells	2.1	1.2	50.1
crude membrane of shocked cells	0.2	0.6	0.4

* EU/gm dry weight of cells

The dry weight of cells was 0.18mg/OD_{600}/ml.

TABLE VIII. The release during the sphaeroplast formation of *E. coli* ML308.225 of adenylyltransferase compared to marker enzymes.

Cell fraction	Enzyme activity *		
	AAD-(3")	Alkaline phosphatase	Lactate dehydrogenase
cell-free sonicate	16.7	16.5	651
sphaeroplast supernatant	1.9	16.9	44
nucleic acid digest supernatant	3.0	0.5	132
sonicated sphaeroplasts	10.3	0.9	430

* EU/gm dry weight of cells

The dry weight of cells was 0.16mg/OD_{600}/ml.

effects that both had on the activity of the enzyme under the specified conditions. Adenylyltransferase was virtually unaffected by SITS prior to sonication though the activity could be reduced by 40% after sonication. SITS did not inactivate the very susceptible lactate dehydrogenase in whole sphaeroplasts. 10mM SITS was found to increase the inactivation of adenylyltransferase in cell-free extracts by only 10% (50% in total).

Trypsin could reduce adenylyltransferase activity in sphaeroplasts to 58% of normal. At the same time, internal enzymes were unaffected by the treatment, though susceptible to varying degrees in cell-free extracts. Adenylyltransferase was very sensitive to trypsin action in cell-free extracts. Trypsin inhibitor and trypsin-inhibitor complexes, in comparable concentrations, had no effect on the activities of any of the enzymes.

15. Comparative effect of R71-a on the DHS susceptibilities of E. coli K12 strain 7 and a mutant derivative E. coli K12 NR-70.

E. coli K12 NR-70 is a mutant of strain 7 with a reduced sensitivity to streptomycin. R71-a was introduced into the two strains to see what relative effects it had on the MICs for DHS for each. The alterations in MICs after the aquisition of R71-a are presented in table X.

The R+ derivatives of the two strains were clinically resistant to streptomycin. The difference in MICs demonstrable prior to the receipt of the R factor was again evident in the R+ strains. The difference was maintained in lieu of the fact that the Kms and the specific activities of the enzymes extracted from each were the same. The graphical determinations of the respective Kms are presented in the fourteenth figure. Enzyme for these calculations was obtained from osmotic shockates that had been concentrated 5X in a 10ml Amicon filter apparatus with UM10 filters (excludes proteins 10,000MW and over).

Table IX. Effect of the chemical treatment of E. coli ML308.225 R71-a sphaeroplasts with SITS and trypsin on adenylyl-transferase and marker enzyme activity before and after sonication.

Reagent	Treatment**	Enzyme activity*		
		AAD-(3")	Lactate dehydrogenase	Uridine phosphorylase
SITS	A	4.2	4.4	ND
	B	6.5	212	ND
	C	7.0	212	ND
Trypsin	A	1.2	242	309
	B	7.8	343	611
	C	13.5	320	570
	C+inh	12.6	320	556
	C+inh.enz	12.6	306	574

* EU/gm dry weight of cells

** A : treatment after sonication, B : treatment before sonication, C : control, C+inh : control plus inhibitor alone, C+inh.enz : control plus enzyme-inhibitor complex alone.

ND : not determined

TABLE X. Effect of R71-a in an E. coli strain that exhibits reduced aminoglycoside accumulation (and reduced aminoglycoside sensitivity) on DHS susceptibility as compared to the wild-type parental strain.

Strain	DHS MIC ug/ml	Adenylyltransferase sp. act. EU/mg prot	Km (M)
K12 Strain 7	0.03	-	-
K12 NR-70	0.24	-	-
K12 Strain 7 R71-a	3.9	4.1×10^{-2}	2.5×10^{-5}
K12 NR-70 R71-a	15.6	3.8×10^{-2}	2.5×10^{-5}

- : no activity

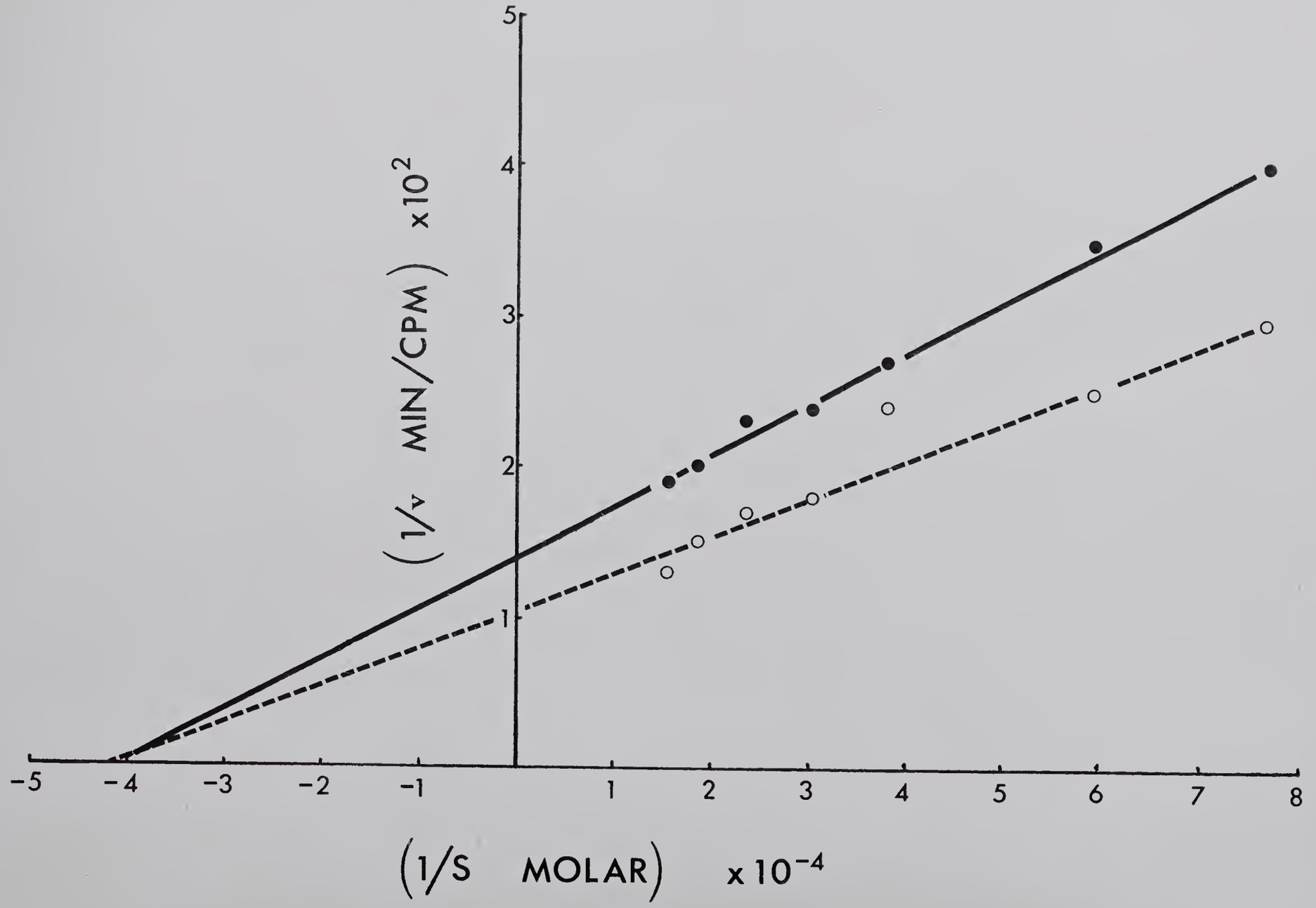

5
4
3
2
1
(1/v MIN/CPM) x10^2
-5
-4
-3
-2
-1
1
2
3
4
5
6
7
8
(1/S MOLAR) x10^-4

FIGURE 14: Graphical determinations of Km values, from Lineweaver-Burk plots, for streptomycin adenylyltransferase activities in osmotic shockates of *E. coli* K12 strain 7 (O- - - - -O) and *E. coli* K12 NR-70 (●———●) with R71-a.

DISCUSSION

The biochemical characteristics of naturally isolated bacteria accounting for their resistance to antibiotics by virtue of R factor-mediated functions have been described with respect to several antibiotics (Benveniste and Davies, 1973). As yet it is poorly understood what the mechanisms of R factor-mediated sulphonamide and tetracycline resistances are, though in the latter case resistance has been suggested to be linked to a decreased uptake of the drug perhaps with the involvement of an inducible transport inhibitor. Otherwise, R factor-mediated antibiotic resistance is apparently the result of the enzymatic modification of the drug resulting in inactive products. Resistance to the penicillins, chloramphenicol and the aminoglycosides is achieved in this manner. Penicillin resistant, Gram-negative and -positive organisms have been shown to possess beta-lactamases, a family of enzymes responsible for the hydrolysis and inactivation of penicillins. Since their discovery beta-lactamases have been extensively studied with regard to their involvement in resistance. Chloramphenicol resistance in clinical isolates has been associated with a plasmid determined acetyltransferase which can also be found in both types of Gram-staining bacteria. The 3-acetyl and 1,3-diacetyl ester derivatives of chloramphenicol lack biological activity. Efforts at detecting aminoglycoside inactivation by bacterial isolates have resulted in the characterization of a large number of modifying enzymes. The basic mechanisms of inactivation were previously mentioned in the Introduction.

Laboratory observations bring to the forefront a pertinent dissimilarity between the resistances to aminoglycosides and the two antibiotics, penicillin and chloramphenicol. In the latter cases R+ cells

have the capacity to inactivate completely the drug medium within which the growth of the cells is permitted (Davies and Benveniste, 1974 and Lundbäck and Nordström, 1974a). As a consequence there are definite inoculum effects when determining levels of resistance. For the aminoglycosides no such detoxification has been observed and the resistant bacteria continue to multiply in what would normally be considered a bacteriocidal medium. Inoculum effects are not generally seen. An alternate explanation for the mechanism of resistance in these strains has recently been advanced based on the following observations. A direct relationship, in R+ strains, between antibiotic susceptibility and cell-free enzyme activity exists (Benveniste et al, 1970). Aminoglycoside susceptibility is dependent on the ability of organisms to actively accumulate drug (see Introduction). Preliminary investigations reported by Bryan and Van Den Elzen (1975) have indicated that in R+ strains there is indeed an R factor-mediated reduction in energy-dependent accumulation of aminoglycosides in E. coli and P. aeruginosa. From these cells could be recovered modified drug amounting to less than 1% of the added antibiotic. Davies and Benveniste (1974) hypothesized that the small amount of accumulated and modified drug would act to block the further uptake of drug. This would spare the cell the necessity to inactivate the drug medium, a feat considered to be beyond the capacity of these enzymes anyway in view of their comparatively low specific activity (Lundbäck and Nordström, 1974a), and in turn spares the demand that would be made on energy (ATP) stores. An assumption of this hypothesis would be that the drug, upon being inactivated, would remain associated at a membrane site thereby enriching the membrane with modified products whereupon interruption of transport would be effected.

A model system composed of E. coli strains resistant to dihydrostreptomycin by virtue of possessing an R factor-determined streptomycin adenylyltransferase (AAD-(3")) was employed to test this hypothesis and its assumptions as well as to confirm and further elucidate the enzymes physical and functional relationship with the bacterial cell membrane.

Inferences of the cellular localization of AAD-(3"), and aminoglycoside-inactivating enzymes in general, have largely been drawn from indirect experimentation such as through the comparative release of enzymes by osmotic shocking and sphaeroplast formation. AAD-(3") can be released from all the E. coli strains tested here by osmotic shocking. The release is quantitative as it is for the periplasmic enzyme alkaline phosphatase (table VII). Beta-galactosidase activity, as an internal marker, is indicative of the release of cytoplasmic components and can be seen to be found in the shockate fluid to a level 40% of that in cell-free sonicates. This is generally in excess of amounts reported to be released during this procedure (Neu and Heppel, 1965), where as little as 5% had been detected. Nonetheless the two populations of protein are clearly distinguishable. Davies and Benveniste (1974) have previously reported, and it is confirmed here (table VIII), that AAD-(3") is quantitatively retained by sphaeroplasts of R+ cells, compared to controls, when at the same time there is a release of alkaline phosphatase into the suspending medium. The release of some AAD-(3") activity during the procedure can be attributed to sphaeroplast disruption or membrane leakage as there is a parallel release of the cytoplasmic marker. In this manner the location of AAD-(3") can be shown to be distinct from the marker enzyme populations. To be bound by sphaeroplasts yet not cytoplasmically located suggests a membrane association of the

enzyme. However, for the data collected from work involving sphaeroplasts to be acceptable two considerations must be dealt with. In the stripping of wall components from the cell would the site of the enzyme be altered and might the enzyme be retained by sphaeroplasts in association with contaminating elements of the cell wall such as polar caps? The first problem would most likely affect the interpretation of the experiments of table IX to be discussed later. In answer to it, it is evident that the enzyme must occupy the same functional site in sphaeroplasts as the uptake of DHS in these preparations was identical, in qualitative terms, to that in whole cells. There still was mediated in the R+ sphaeroplasts a reduction in accumulated drug. With respect to the second question sphaeroplasts were prepared from a strain of E. coli well documented to produce sphaeroplasts lacking substantial cell wall components when the prescribed methodolgy is applied. It has been noted that in the present work no contaminating elements of the cell wall were visible by electron microscopic examination.

An association of the enzyme with the membrane cannot be directly inferred as attempts to detect enzymatic activity with isolated membrane preparations (Kaback vesicles) have failed. The enzyme does not appear to require associated lipid for activity since the treatment of crude enzyme preparations with Triton X-100 has no deleterious effect. It is therefore distinct from the typical membrane enzymes (intrinsic protein) in this concern (Rothfield and Romeo, 1971). At best the relationship with the membrane can be considered a tenuous one.

The simple structural compartmentalization of bacteria is the basis of difficulties in localizing the so-called soluble proteins by cell fractionation. Methods have been developed for use in the study of the

localization of periplasmic proteins in the absence of cell fractionation (Heppel, 1971). Some of these have been successfully applied to the study of binding proteins (Rosen and Heppel, 1973) most of which are periplasmic and, in some authors view (Oxender and Quay, 1975), have some affinity for the cell membrane (they are generally released during sphaeroplast formation so the association with the membrane is essentially inferred on the basis of functional considerations). In some instances reagent dyes have been applied in these studies (Pardee and Watanabe, 1968).

SITS (4-acetamino-4'-isothiocyanotostilbene-2,2'-disulphonic acid) was found not to inactivate a number of periplasmic enzymes in cell-free systems so this precluded the use of whole cells in the experiments of table IX as no controls could be established. Highly charged as it is, SITS would not be expected to penetrate plasma membranes and therefore would only have access to proteins exterior to such permeability barriers. This reagent dye reacts, apparently covalently, with amino, histidyl and guanidyl groups of protein (Maddy, 1964).

Little or no inactivation of AAD-(3") by SITS occurred in sphaeroplasts. Binding may have occurred but not at a critical site (active site?) of the molecule. Trypsin, on the other hand, can inactivate the enzyme presumably because its action, in the form of a peptide cleavage, anywhere on the molecule could indirectly affect the active site of the enzyme by inducing conformational changes in the whole molecule. The enzyme, however, is not affected to the extent that it is in cell-free systems. The reduced sensitivity in sphaeroplasts can be accounted for by the protection in stereochemical terms offered by an association with the membrane. The absence of inactivation by SITS may be indicative of

an obscured active site. In this event a molecule of similar molecular weight, such as dihydrostreptomycin, may also be denied direct access. The charge properties of the two molecules are quite different and this is another consideration to be made in this respect. The susceptibility of AAD-(3") to trypic action in sphaeroplasts when internal marker enzymes are not affected indicates that the enzyme or some fraction of it lies outside the membrane permeability barrier.

Characteristically the aquisition of R71-a by E. coli results in a form of low-level resistance (table I). The resistance is reflected in a clear reduction of DHS accumulation after thirty minutes at the drug concentrations tested (figures 4 to 7). Bacteria resistant because of single-step chromosomal mutations affecting ribosomal affinity for DHS have also been shown to actively accumulate reduced amounts of drug (Bryan and Van Den Elzen, 1976). The R+ cells of this study have ribosomes quite competent to binding DHS (table II). The adenylylating enzyme appears to be the determining factor in reducing accumulation. This is substantiated using a mutant of an E. coli strain with the R factor. The mutants were more susceptible and accumulated more DHS than did the parent. The evidence indicates the mutation involved only a reduction in the amount or activity of the adenylyltransferase judging from comparative activities in cell-free preparations (table III). The following observations support this conclusion. Firstly, EMS was selected for the mutagenesis on the basis of its ability to cause point mutations (transitions) and not deletions (Osborn et al, 1967). Secondly, the mutation also affected spectinomycin susceptibility and the enzyme also mediates resistance to this antibiotic (Benveniste et al, 1970). Transport systems for the two drugs are different though not necessarily distinct (Bryan and Van Den Elzen, 1977). Mutants

affecting the transport of one need not affect the transport of the other. Lastly, low-level resistant revertants could be isolated at high frequency supporting the assumption that only a single mutational event was responsible.

The reduction in accumulation seen in R+ cells does not occur generally throughout the entire uptake profile. Typically, uptake occurs in three stages in sensitive cells (figure 4) and these have been thoroughly investigated by others (Bryan and Van Den Elzen, 1977). The first phase represents a rapid binding of drug to outer wall components. In figure 5 it can be seen to occur in KCN poisoned cells as well. It is energy-independent. The reversible binding of this population of drug is evident in figure 10 as that label lost within two minutes of the resuspension of prelabelled cells into unlabelled DHS. In the same figure there can also be seen a second population of drug which is lost slowly from poisoned cells but not the untreated ones. Where cells maintain the capacity to actively accumulate drug this second population of reversibly bound drug can be internalized or irreversibly bound. Though seen in this case only in R+ cells, it may represent the ionic binding of DHS within the deeper layers of the cell wall such as the surface of the membrane from which it can be actively accumulated during subsequent events without being lost from the cell. In other words, internalization of drug from this site would exceed the rate of dissociation. The following two phases of uptake are absent in the KCN poisoned cells and represents drug that has been accumulated actively. In sensitive cells the first energy-dependent phase is characterized by a low rate and is concentration dependent. It is followed by a third phase of greatly enhanced rate. R+ cells at certain concentrations of

drug (figure 4) differ from sensitive cells in their DHS accumulation profiles by not demonstrating this second energy-dependent phase. The differences in total uptake of DHS between sensitive and resistant cells can be attributed to the absence or the delayed onset of a third phase of accumulation which, when it does exist, is at a lower rate when compared to that of sensitive cells at the same drug concentration. As observed during the interim of this study and elsewhere (Bryan and Van Den Elzen, 1977) ribosomally resistant bacteria similarily fail to accumulate drug during this final phase. The second phase of uptake progresses normally in these strA mutants. In whole cells (figure 4) and in sphaeroplasts (figure 8) of R+ and R- bacteria the rates of secondary phase uptake are also identical.

The absence of the third phase in ribosomally resistant bacteria must be attributed to the inability of the ribosomes to bind antibiotic. Direct effects of strA mutations on membrane transport functions have never been documented. Their only known effect is that associated with the ribosome. It is hypothesized that in sensitive cells drug is transported across the cell membrane to the ribosome thus constituting the second phase of uptake. Binding of the drug by the ribosome initiates the transition to third phase kinetics by altering the status quo of membrane energy transduction somehow, such that more energy is made available for aminoglycoside transport (Bryan and Van Den Elzen, 1977). Therefore, in strA mutants the failure to bind drug is responsible for the absence of third phase kinetics. It is the onset of this phase that signals the first signs of protein synthesis inhibition followed by cell death (Bryan and Van Den Elzen, 1977). Instead, drug would continue to be accumulated by second phase kinetics until the cellular binding sites

became saturated as has been shown to occur by Bryan and Van Den Elzen (1977). Cells capable of modifying aminoglycosides could conceivably transport the drug normally to the target site (ribosomes), as is expected with strA mutants, but binding would not occur due to the drugs inactivation (Chang and Flaks, 1972). The evidence provided suggests indeed this is the explanation for the absence of third phase kinetics in R+ cells and likewise, the absence of bacteriocidal effects.

The peptidoglycan layer, but not the outer membrane-LPS complex, of the cell wall represents a partial barrier of some form to the penetration of aminoglycosides in E. coli and P. aeruginosa (Tseng and Bryan, 1974 and Bryan et al, 1975). However, the second uptake phase is the rate-limiting step in aminoglycoside transport and this is considered to involve the transport of the drug across the cell membrane (Bryan and Van Den Elzen, 1977). Uptake experiments using sphaeroplasts bear this out in qualitative analysis. Accumulation profiles are similar in R- sphaeroplasts and whole cells to the point that the transition to the third phase occurs at the same point in time (figures 7 and 8). The absence of the cell wall did not apparently influence the transport rate of the second phase. The enhanced streptomycin uptake observed in cells treated with carbenicillin must therefore be expressed during the third phase. The absence of this phase in the sphaeroplasts of R+ cells indicates that the cell wall plays no significant protective role in the resistance of these organisms.

R+ cells growing in the presence of dihydrostreptomycin do not release adenylylated drug into the growth medium (figure 9). No modification or obvious loss of label is apparent compared to controls. At the same time there is no efflux, or cycling out of the cell, of actively

accumulated drug, modified or unmodified (figure 10). At the same time when sensitive cells enter the third phase of uptake R+ cells have actively accumulated an equal amount of drug (figure 7). R+ cells continue to accumulate drug at the same rate so that after thirty minutes they contain five times as much antibiotic than did the sensitive cells at the moment of entry into the final phase. At the transition point sensitive cells have achieved a ratio of one molecule of drug per twenty ribosomes assuming there to be 20,000 ribosomes per cell and 2×10^9 cells per OD_{600} unit/ml. During the second phase of accumulation in R+ cells, the only energy-dependent phase observed at certain drug concentrations (figures 4 and 7), adenylylated dihydrostreptomycin is being taken up by the cells (figures 11 and 12). After thirty minutes roughly 40% of the cell-associated drug is modified. This percentage is in good correlation with the amount of drug that would be expected to have been actively accumulated at this time. The cell-bound and unmodified DHS would represent non-specifically, reversibly bound drug.

The intracellular distribution of the adenylylated dihydrostreptomycin in R+ cells does not differ from the distribution of DHS accumulated by sensitive cells on the basis of the tests performed here. In both instances label from fractionated cells can be essentially localized in soluble fractions with very little being found in isolated membrane preparations (tables V and VI). There is no apparent enrichment of label in the membranes of R+ cells. Autoradiographic analysis of cells having accumulated DHS for the same period of time indicates that there is no qualitative difference in the distribution of drug in R+ and R- cells (figure 13). A moderate tendency to an internal localization is observed in each case. One would hypothesize that a

similar profile could be achieved using strA mutants however this, as yet, has not been attempted. Obviously, from this data the majority of label is membrane and/or cell wall associated. The inability to localize label to membranes by fractionation may, in fact, be indicative of the relatively weak binding to this structure. In all, there is no evidence to suggest that modified drug blocks transport by taking up a permanent site in the membrane.

Several authors have observed that the efficacy of R factors involved in determining aminoglycoside resistance was dependent upon the permeability properties of the bacterial strain. Bryan et al (1975) found that differences in MICs were retained in the R+ derivatives of two different P. aeroginosa strains in spite of the two having modifying enzymes of the same Km and specific activity. Lundbäck and Nordström (1974b) introduced an R factor, coding for a streptomycin adenylyltransferase, into an E. coli mutant that accumulated less streptomycin than the parent to discover that the resulting level of resistance was ten times greater than that of the parent strain with the R factor. The R- derivatives of the strains demonstrated the same ten-fold difference in MICs albeit at lower concentrations of drug. The mutation and the R factor were consequently synergistic with respect to determining streptomycin resistance. Combined with the low activity characteristic of aminoglycoside-inactivating enzymes this suggested that there was a competition in cells between the rates of inactivation and transport. To test this, R71-a was introduced into two E. coli strains isogenic but for a mutation in one that acted to reduce the rate of aminoglycoside transport. DHS MICs for the two strains differed (table X) in accordance with this information. The mutation is believed to reduce the supply of energy required for transport by pre-

venting the maintenance of the high energy state of the membrane (Bryan and Van Den Elzen, 1977). Receipt of the R factor by both strains elevated MICs but the mutant strain remained proportionately less susceptible. The specific activities and the Kms of the enzymes from the two bacteria are essentially identical (table X) indicating that the difference in the susceptibilities of the R+ strains is a function of the drug permeability properties of the bacteria. Aminoglycoside susceptibility would appear to be determined by the balance between the rate of inactivation on the one side and the rate of transport on the other. Resistance results when inactivation rates outweigh transport rates so that actively accumulated drug is in the modified form. Consistent with this hypothesis is the observation that a gentamicin acetyltransferase, which inactivates both gentamicin and tobramycin, confers resistance in E. coli only to gentamicin (Biddlecome et al, 1976). This is explained by the fact that the Km of the enzyme for gentamicin is ten times lower, and the V_{max} six times higher, than for tobramycin. Presumably the inactivation rate for tobramycin cannot compete with the rate for aminoglycoside transport in these bacteria.

Based on the evidence presented a sequence of events can be hypothesized to occur in R+ E. coli growing in the presence of dihydrostreptomycin. After an initial rapid binding, DHS is bound fortuitously by numerous, low-affinity membrane binding sites described by Bryan and Van Den Elzen (1977) and represented perhaps by the second population of drug lost from KCN poisoned cells as seen in the efflux experiments of figure 10. From these sites, drug is internalized and inactivated during transport (since inactivated drug can only be found associated with the cell) and inactivated early in the process since the enzyme is

external relative to the membrane permeability barrier. Once inside, in the absence of binding to the ribosomes, the drug accumulates until cellular sites are saturated analogous to the situation observed in strA mutants. In this manner the cell only inactivates a fraction of the total antibiotic present in the medium. In conclusion, resistance to aminoglycosides in R+ bacteria arises because the drug that is actively accumulated is inactivated and fails to bind to the ribosome, thereby failing to initiate the events leading to cell death. It is also significant that the rate of drug inactivation exceeds the rate of drug transport.

Little can be offered to the discussion concerning the bacteriocidal effects that streptomycin and other aminoglycosides have on the cell based on this work. One observation, that DHS could induce the lysis of sphaeroplasts, is consistent with the theory that cell death is the result of gross alterations to the membrane's integrity. As a final note, efforts to overcome the aminoglycoside resistance of R+ bacteria might well be worthwhile if directed at finding agents capable of accelerating transport so that enzymatic inactivation is no longer competitive. The clinical significance of this form of resistance is presently dependent on the rather low rate of aminoglycoside transport of most bacteria. Success would be accompanied by the option to reduce dosage levels to a point where the risk of toxic reactions was eliminated. In this direction much is presently being achieved in the definition of the energetics and mechanisms of aminoglycoside transport in bacteria.

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